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UNIVERSITY OF ALBERTA

Subspecific Variation within the d-Loop Region of Mitochondrial DNA of Bison (*Bison bison*) and Wapiti (*Cervus elaphus*)

by

Renee O. Polziehn

A Thesis

Submitted to the Faculty of Graduate Studies and Research

in Partial Fulfilment of the Requirements for the

Degree of Master of Science

Department of Zoology

Edmonton, Alberta

Fall, 1993

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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled 'Subspecific Variation within the d-Loop Region of Mitochondrial DNA of Bison (*Bison bison*) and Wapiti (*Cervus elaphus*)' submitted by Renee O. Polziehn in partial fulfilment of the requirements for the degree of Master of Science in Zoology.

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Abstract

Presently under debate in North America is the putative subspecific status of the Manitoban wapiti (*Cervus elaphus manitobensis*), Rocky Mountain wapiti (*C. e. nelsoni*), Roosevelt wapiti (*C. e. roosevelti*), wood bison (*Bison bison athabascae*) and plains bison (*B. b. bison*). To assess the genetic distinction of these subspecies, molecular techniques were employed to assay variation in the d-loop region of mitochondrial DNA. The d-loop region was amplified using the polymerase chain reaction, restricted with endonucleases, and inspected for restriction fragment length polymorphisms (RFLPs). A new technique was developed which employed primers with template-dependent restriction sites that could assay eight additional polymorphic sites identified from sequence information. This method provides less information than sequencing, however more individuals can be surveyed. Sampled from Olympic Peninsula National Park, Strathcona Provincial Park, Kootenay National Park, Banff National Park, Rocky Mountain House, Duck Mountain Provincial Park, and Elk Island National Park (EINP) were 49 wapiti. Sampled from Mackenzie Bison Sanctuary, EINP; Custer State Park (CSP), Ft. Niobrara Wildlife Refuge, National Bison Range, Wichita Mts. Wildlife Refuge, Yellowstone National Park, and Wood Buffalo National Park (WBNP) were 269 bison. The three and 11 haplotypes identified among the wapiti and bison populations, respectively, were used to evaluate variation within and between populations. The frequency of haplotypes was variable among all populations, presumably as a result of genetic drift and variation in founding members. Haplotypes found among wapiti were as follows; Roosevelt wapiti were one type, Manitoban and most Rocky Mountain wapiti were a second type, and few Rocky Mountain wapiti were a third type. This partitioning of

Mountain wapiti were a third type. This partitioning of haplotypes suggests wapiti are defined by at least two subspecies. Among bison, one haplotype showing a close resemblance to domestic cattle (*Bos taurus*) was found in samples from CSP; and four haplotypes were found exclusively in bison originating from WBNP. These haplotypes, however, did not separate bison into wood or plains. Phylogenetic analysis of the bison haplotypes, using the computer program PAUP, indicated a paraphyletic lineage for plains bison and polyphyletic lineage for wood bison, suggesting that there are no well defined subspecies of bison.

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Chapter 1

General Introduction

Establishing the genetic integrity of a taxon plays a significant role in determining its future. Classification affects management decisions, and moreover, legal position and entitlement to protection (Geist 1992). Under the Wildlife Act passed in Canada in 1973, protection, for purposes of conservation and biodiversity, can be granted to a species, subspecies, or population. Concerted efforts to protect the subspecies of bison known as wood bison (*Bison bison athabascae*), have moved the population from an endangered to a threatened state. Wapiti (*Cervus elaphus*) populations, although they are not excessively small, also depend on their classification for protection.

The taxonomic status of bison and wapiti subspecies, however, is currently under debate. The subspecific status of wood bison in northern Alberta was first questioned in the late 1800's (Rhoads 1897), years before the national park called Wood Buffalo (WBNP) was established to protect the population. The issue rested until the 1920's when plains bison were introduced into WBNP, a controversial decision that led to the creation of 'hybrid' bison. The subspecific status of wood bison recently received attention when the 'hybrid' bison in WBNP were threatened to be replaced with pure, healthy wood bison from Elk Island National Park: bison that descended from an isolated herd at WBNP after plains bison (*B. b. bison*) were introduced. The wood bison issue also raised questions regarding the subspecific status of wapiti in North America because wapiti have also been manipulated and translocated throughout the continent over the past 100 years.

To understand the taxonomic relationship between subspecies, comparisons are typically drawn between the geographic distributions and morphological, behavioral, or physiological characters. The following overview outlines the introduction, establishment and localization of bison and wapiti in North America, and describes the morphological and present molecular approach used to distinguish their subspecies.

1.0 Introduction, Establishment and Localization of Bison and Wapiti

Most large mammals found in North America today first appeared in Asia at the beginning of the Pleistocene or Ice Age (12 - 1 million Y.B.P.; Guthrie 1966). During the Ice Age, species from Asia expanded into northern Siberia, crossed the Bering-Chukchi land bridge, and dispersed across North America. The land bridge, which first appeared between these two continents around 36 million Y.B.P. (Oligocene), has been intermittently inundated and exposed since then (Bryant and Maser 1982). During the last interglacial period between 40,000 - 28,000 Y.B.P., the land bridge became impassable (McHugh 1972). The bi-directional passage was again re- established for a brief period between 28,000 - 10,000 Y.B.P. (Bryant and Maser 1982). Consequently, the separation of North American and Eurasian mammals has been recent (Bryant and Maser 1982; Van Zyll de Jong 1986).

Both ancestors of bison (*Bison bison*) and wapiti (*Cervus elaphus*) evolved in the Old World, dispersed across North America, and became divided into apparently distinct subspecies with respect to geography (Guthrie 1970; Bryant and Maser 1982). Evidence of the movement of bison and wapiti across the natural causeway is supported by fossils found in glacial refugia from the Illinoan period (300,000 Y.B.P.; Geist 1971). Vertebrates, including bison and wapiti, moved gradually and repeatedly across

the Bering-Chukchi platform, and are believed to have been continuous with Eurasian populations (Bryant and Maser 1982).

While animals were moving freely across the Bering land bridge, most of northern North America was covered with the Laurentide and Cordilleran glaciers, and considered inaccessible until the Holocene (10,000 years to present) (Murie 1951; Pielou 1991). Animals, however, moved through ice-free corridors along the Rocky Mountains or Pacific coast to open ranges in central and southern regions of the continent. In 1947, fossils of bison (*B. latifrons*) and other ungulates from the beginning of the Pleistocene were uncovered in a creek bed in southern Kansas (McHugh 1972). The Rocky Mountain corridor periodically closed during the Pleistocene, with the last restriction to movement occurring from 23,000 to 13,000 Y.B.P. (McHugh 1972). The Pacific corridor was also intermittent and consisted of numerous ice-free refugia along the shores and on the offshore islands of present day Alaska and British Columbia. These refugia along the west coast acted as stepping stones in the migration of mammals, including bison and wapiti, at a time when Rocky Mountain corridor was blocked by ice (Pielou 1991).

Animals dispersed in ice-free areas of North America were subjected to diverse environmental pressures during the Pleistocene. Fluctuations in climate during this period greatly modified terrestrial habitats and produced vegetational changes (Geist 1983). Because ungulates are particularly sensitive to vegetational changes, the environmental fluctuations are believed to have contributed to ungulate phenotype and genotype diversification (Geist 1971).

The retreat of the glaciers during the Holocene presented an opportunity for wapiti and bison to expand further into new habitats. This expansion could have resulted in populations of one phenotype or monotype being established. The formation of specialized ecological regions such as the Great Plains, however,

demanded parallel specialization of populations, and from this pressure arose polytypic species. As populations expanded across North America, they became established in the ecozones and over time, their phenotype would have reflected the conditions of each region. Localized grazing areas would have served to isolate populations, and to reduce gene flow (Bryant and Maser 1982) because animals generally move between the same summer and winter ranges (Soper 1941; Van Camp 1989).

Movement of bison and wapiti is largely in response to seasonal availability of suitable habitats, in particular to abundance of food (Peek 1983). Migratory movements of bison from the prairies north into the southern range of the boreal forest (Seton 1886) likely reflects the greater range requirements of plains animals. Although the prairies provided excellent habitat for ungulates, the grasslands were subjected to intense grazing pressures when large herds of bison moved into the area. This grazing pressure forced animals to respond by moving across the prairies until the grasses recovered. Therefore, bison from the prairies were forced to migrate larger distances and across more vegetational zones than the northern bison populations.

Other factors that influence ungulate dispersal include predation, disease, weather, population density, and mating strategy (Gates and Larter 1990). For example, males become more nomadic as the rutting season advances, while females tend to be more stationary (Gates and Larter 1990).

Although the bison and wapiti populations generally remained within their respective vegetation zones, their movements were not entirely restricted by geographic barriers. Herds of both bison and wapiti, or their skeletons, were sighted in mountain passes by settlers and hunters in the late 1800's (Dary 1974; Murie 1951). The montane and intermontane bison populations were first thought to represent a separate species, although they were later identified as plains bison that had penetrated the mountains via the river valleys (Van Zyll de Jong 1986). The American Bison Society,

created in 1905, also regarded the distinction as no more than a climatic or geographical variation, and concluded that 'mountain bison' were not distinct from other bison (Dary 1974). Geist (1971) speculated that wapiti in the Rocky Mountains were hybrids resulting from montane and coastal refugia, while Murie (1951) and Guthrie (1966) suggested that subspeciation occurred once the populations had moved into their present range. Although movement across mountain ranges was not impossible, it was never extensive. Fossil records for wapiti along the Pacific coast suggest they had little or no overlap with interior populations (Bryant and Maser 1982).

In areas where geographic barriers do not exist and cannot act to decrease the migration and integration of individuals between populations, less obvious barriers may be in place. On the microgeographic level, behavioral barriers have been found to support genetic subdivision in cotton rats (*Sigmodon hispidus*, Kessler and Avise 1985). Invading individuals will be unfamiliar with the new range and this can affect their predator avoidance and foraging ability. Immigrants may also have to establish a new position in the social structure of the new population. These behaviors will decrease the reproductive success of immigrants and their chance of becoming integrated with the native population.

The movement of animals between each others' territories establishes a transition zone, but does not necessarily establish gene flow (Van Camp 1989). The transition zones are considered to be hybrid zones only when genetically distinct populations meet, mate, and produce hybrids (Barton and Hewitt 1989). If the hybridization produces less fit offspring, a steep or discontinuous cline will form between the populations (Van Camp 1989). In comparison, a positive or neutral effect on fitness will broaden the hybrid zone until all subpopulations become genetically undifferentiated (Van Camp 1989). Because most transitional areas coincide with vegetative transitional zones (Van Zyll de Jong 1986), populations inhabiting the

parkland area would likely have had genetic affinities with both boreal and prairie populations (Blyth and Hudson 1987).

1.1 Morphological Approaches to Subspecies Identification

Although large, circumpolar mammals, including wapiti, moose (*Alces alces*), bison, and caribou (*Rangifer tarandus*), exhibit morphological variation, populations cannot always be distinguished from each other based on the absence or presence of any one morphological feature (Guthrie 1966; Van Zyll de Jong 1991). Pelage, one of the more commonly used taxonomic characters in mammals, has led to taxonomic confusion when account was not taken of age- and sex-related differences, seasonal changes due to rapid hair growth, wear, seasonal moulting (Geist 1991), and intra-population variation (Murie pers. com. 1993). Characteristics of bison, such as body weight and horn size, also depend on factors such as age, sex, season, general health, nutrition, and population density (McHugh 1972; Berger and Peacock 1988). Even the replacement of crown-like or coronate antlers of North American wapiti, found in the warmer oceanic phases of the Ice Age, by acoronate forms during the cold continental phases, was apparently caused by environmental changes (McHugh 1972).

The description of a subspecies was often based on the coloration and measurement of external morphology from only one animal (Bryant and Maser 1982; Gavin 1988), and this would lead to problems of classification as records used to describe and identify taxa often lacked the information to discriminate between slight morphological variations within and between subspecies.

In the last 30 years, studies to establish the subspecific status of a population have moved from morphology to morphometry. Morphometry can detect inter-population variation, but the measurements are still influenced by variation in an animal's diet, health, and environment. Separating subspecies of bison and wapiti on

ratios derived from cranial measurements is not considered possible because each measurement has a high degree of plasticity and variability (Bayrock and Hillerud 1964; Hutton 1972; Shackelton *et al.* 1975). Cranial, horn, and limb measurements of bison subspecies indicated only a north-south cline effect (McDonald 1981). This conclusion was contested by Van Zyll de Jong (1986) who used skull ratios from bison, in addition to pelage color, presence or absence of a cape, bonnet, or chaps, the location of the longest spine of the thoracic vertebra, and horn measurements to suggest the subspecies were genetically different. Likewise, morphometric analysis of body size parameters for Rocky Mountain, Manitoban, and Roosevelt wapiti by Blood and Lovaas (1966), McCullough (1966), and Troyer (1960) suggested that at least three definite forms of wapiti exist.

More morphometric evidence separating the existence of subspecies may have been found had populations of bison and wapiti remained undisturbed following their establishment into ecozones. Wapiti were eliminated from the eastern states and provinces, and bison were pushed to near extinction by heavy demands for hides and meat following the introduction of European settlements along the eastern coast of North America in the 1600's. Seton estimated about 10,000,000 wapiti and 75,000,000 bison were present in North America before the arrival of Europeans (Murie 1951; Dary 1974). Fuller (pers. comm 1993) estimated the original number of bison to be closer to 15 million. By 1907, less than 100,000 wapiti (Bryant and Maser 1982) and 1,000 bison (Jenning and Hebbing 1983) were estimated to exist. Wapiti and bison populations had declined at an alarming rate. Records of one person shooting 3,000 bison a month or 250 a day were noted by Roe (1951) and Christiansen (1991). These records can be compared with General Sibley's estimates in 1854 which placed figures at 250,000 to 500,000 bison killed per annum during the period between 1830 and 1860; numbers which were considered to be conservative (Garretson 1938).

It was not until North America became nearly devoid of these two species that legislation for the protection of wildlife was passed.

Separating extant bison or wapiti subspecies on the basis of morphology and morphometry was also complicated by efforts to conserve these species because many populations were created artificially, or were derived from hybridizations of local and transplanted animals. For example, Ontario received two introductions of wapiti: Rocky Mountain wapiti were introduced in the 1930's from Jackson Hole, Wyoming and Manitoban wapiti were introduced between 1972 and 1973 from Elk Island National Park, Alberta (Bryant and Maser 1982). Unfortunately, population censuses from the 1940's also raised the possibility that members of the Eastern wapiti had survived and hybridized with animals introduced in the 1930's (Bryant and Maser 1982). Between 1917 and 1920, several hundred Rocky Mountain wapiti were obtained from Yellowstone National Park, Wyoming to supplement western Alberta. Rocky Mountain wapiti and Manitoban wapiti also were introduced to the Banff National Park area of southwestern Alberta in the 1930's and 1970's (Bryant and Maser 1982). 54 Rocky Mountain wapiti from Jasper National Park and 46 from Elk Island National Park were also moved to Manning, Alberta in 1972-1973. Since then, wapiti from Elk Island National Park have also been moved to northern Saskatchewan and to the eastern slopes of the Rocky Mountains.

Similar events have occurred at Wood Buffalo National Park which straddles the Northwest Territories and northern Alberta. Wood bison in this area were believed to be the last survivors of their kind and were placed under protection in 1894. In 1922, the area was established as Wood Buffalo National Park (WBNP) (Northern Diseased Bison Panel 1989). During this same time period, plains bison were given refugee in Buffalo Park near Wainwright, Alberta. By the mid 1920's, the plains bison had exceeded the carrying capacity of their range, and 6,673 surplus plains bison were

sent by rail and barge to WBNP (Novakowski 1989). Fortunately, in 1957 an isolated herd of approximately 200 wood bison was discovered in the northwestern section of WBNP. They were believed to represent the original wood bison because of the relatively few bison trails leading into their area (Novakowski 1989).

During the early and middle 1960's more than 100 of the isolated wood bison from WBNP were captured in two separate winter round-ups and placed into isolation (Dary 1974). A few of the isolated bison were removed and used to found the new wood bison populations at Elk Island National Park in central Alberta and the Mackenzie Bison Sanctuary in the Northwest Territories.

1.2 Molecular Approaches to Identify Subspecies

Until recently, traditional methods of differentiating subspecies were preferred over molecular approaches because many morphological features were easily observed in the field, whereas observations of genetic characters required time-consuming laboratory analysis. Morphological, physiological, and behavioral characteristics, however, 1) are difficult to measure, 2) are influenced by the environment, 3) rely on the expression and interaction of gene products, and 4) depend on the scrutiny of the observer to differentiate between phenotypes.

The possibility now exists for taxonomists to eliminate environmental factors and simply look at the genetic information encoded in the DNA which is not directly influenced by factors such as temperature, humidity, or food supply. Focusing on the genotype, however, does not imply the environment does not place selective pressures on the genetic component. The relatively recent development of the polymerase chain reaction (PCR) has expedited immensely the number of genetic variants, loci, and individuals that can be surveyed in a population. Because mammals exhibit similar physiological functions and, therefore, possess a similar set of genes (Barton and Jones

1983), comparisons can be drawn between homologous sequences of DNA. Changes in the nucleotide sequence can also be analyzed phenetically and cladistically to provide an estimation of genetic distance and divergence, and to infer evolutionary relationships between individuals, populations, subspecies, or species (Nei 1987; Avise 1989; Bernatchez and Dodson 1991; Cronin 1992).

The first studies to determine genetic differences in subspecies of bison and wapiti concentrated on chromosomes and proteins. Hemoglobin, blood protein analysis, blood typing (Stormont *et al.* 1960; Johnston 1968; Naik *et al.* 1970; Cameron and Vyse 1976; Peden and Kraay 1979; Gyllensten *et al.* 1980; Baccus *et al.* 1983; Dratch 1986), and karyotyping (Wurster and Benirsche 1967; Ying and Peden 1977) were employed to distinguish subspecies from each other, but proved to be insufficient. The lack of detectable variation, however, may have been due to limitations of these techniques. While protein electrophoresis provides a quick and easy method of surveying large populations, separation of proteins with similar charges and molecular weights may not be possible; consequently, these differences will not be detected. As a result, efforts were shifted to information directly encoded in DNA.

Mitochondrial and nuclear DNA analysis (Cronin 1986; Bork *et al.* 1991) initially failed to locate unique differences between either wapiti or bison subspecies. However, the variation found in the allelic frequencies of the growth hormone releasing gene between populations of wood and plains bison indicated some separation of the populations over time (Bork *et al.* 1991). The overall lack of variation in growth hormone genes likely reflects the restrictive influences of strong selection pressures. DNA regions coding for important proteins are allowed few nucleotide changes, as both position and type of nucleotide can alter the amino acid sequence and perhaps protein function (Brown *et al.* 1979). Consequently, the present study focused on observing variation in a less conserved region of the DNA.

Mitochondria, which are cellular organelles that possess their own DNA (mtDNA), lack an efficient DNA repair mechanism (Baum 1992). The absence of the ability to repair DNA leads to a higher mutation rate in the nucleotide sequence of mtDNA. However, selection against change is placed on sequences that are responsible for coding for mitochondrial products. Most regions of the mtDNA are conserved because they produce products that play a role in providing energy for the cell. Because the displacement loop (d-loop) does not produce any products, but controls the initiation of transcription and replication of the mtDNA, it is less conserved. The mutation rate of the d-loop region is about two to ten times (0.08 - 2.0 substitutions/bp/10^6yr) greater than the rest of the mtDNA (0.04 - 0.2 substitutions/bp/10^6yr) and another five to ten fold faster than single copy nuclear genes (0.02 substitutions/bp/10^6 yr)(Brown *et al.* 1979; Moritz *et al.* 1989, Vigilant *et al.* 1989). The d-loop of mtDNA was used in this study because higher rates of mutation increases the liklihood of distinguishing conspecific populations (Brown *et al.* 1986; Vigilant *et al.* 1991).

The recent introduction of PCR technology provided the opportunity to observe variations at the genetic level for populations more easily. PCR involves the production of large amounts of a specific region of DNA. Amplification is achieved through repeated cycles of denaturation of template DNA, annealing of oligonucleotide primers flanking the region, and DNA synthesis (Innis *et al.* 1990). The amplified DNA can be evaluated for variation by sequencing or by 1) digesting the products with restriction endonucleases that recognize and cut DNA at short specific sequences, 2) separating the resulting fragments by electrophoresis, and finally, 3) identifying the restriction fragment length polymorphisms (RFLPs).

The identification of RFLPs leads to the identification of genotypes or haplotypes (single copy of a locus). Analysis of haplotypes within and between

populations can provide valuable information with respect to the genetic structure and inter-relationships of populations (Gilbert *et al.* 1990). Theoretically, each subspecies should express a subset of haplotypes that do not occur in related conspecifics. This diversity of haplotypes within and between subspecific populations can establish the present biogeographic boundaries (Avise *et al.* 1987; Gilbert *et al.* 1990; Thomas *et al.* 1990) and historical distributions by confirming the haplotypes in extant populations and museum specimens (Kessler and Avise 1985; Diamond 1990; Yamagata *et al.* 1990). The populations are likely to differ because the maternally inherited and essentially haploid nature of mtDNA reduces the effective population to 25% of the total population and this makes the effects of random drift more visible (Avise *et al.* 1987). Even hybridization events between populations are likely to be detected from mtDNA several generations after the event, and long after back-crossing would have reduced the chance of detecting the event in nuclear DNA (Baum 1992).

Phylogenetic analysis of mtDNA also has advantages over nuclear DNA. The construction of a phylogenetic tree from nuclear DNA can be complicated by crossing-over events that lead to changes in the reading frame and the presence of more than one allele (Baum 1992). However, for all phylogenetic or gene trees constructed from one genetic locus, Pamilo and Nei (1989) advised using caution when inferring the actual evolutionary pathway of a species. This uncertainty can be reduced by using several loci that have evolved independently from each other.

1.3 Taxonomic Status

Whether the approach used to separate taxa is morphological or molecular, certain requirements must be met before a status is assigned. True species generally exhibit three properties: morphological differences, ecological differences, and reproductive isolation (Bryant and Maser 1982). To be diagnostic, at least one character used to identify the species must be discrete, asexual, fixed within the

species, and absent from conspecifics (Mayr 1971; Baum 1992). Subspecies, however, can overlap in these areas, as defined by Mayr (1971:210):

> "A subspecies is an aggregate of phenotypically similar populations of a species inhabiting a geographic subdivision of the range of the species and differing taxonomically from other populations of the species."

This definition implies that although subspecies are often reproductively compatible and have overlapping ranges with related subspecies, they may have descended from a unique lineage and evolved as an independent group. Because phenotypes may not reflect a population's lineage, variations in the genotype were thought to be more informative for defining subspecies. At first the quantity of nucleotide substitutions in a gene were thought to be sufficient to define the status of a taxon; however, even individuals within the same population differ by hundreds of substitutions (Mayr 1971: 321). Therefore, O'Brien and Mayr (1991) suggested that subspecific status should be determined from the phylogenetic relationship of lineages:

> "Subspecies will normally be allopatric and they will exhibit recognizable phylogenetic partitioning because of time-dependent accumulation of genetic differences in the absence of gene flow. Most subspecies will be monophyletic; however, they may also derive from ancestral subspecies hybridization."

Inferring a species or subspecies tree from a gene tree assumes that each haplotype represents a lineage. If all lineages or haplotypes are grouped together as one group, the group is said to be *monophyletic*. More specifically, a group (such as a subspecies) is monophyletic if all the descendants of a common ancestor, including the most recent common ancestor, constitute the group (Ashlock and Mayr 1991). If not all the haplotypes or lineages derived from the recent common ancestor are included in the group, the group would be considered *paraphyletic* (Wiley 1981). *Polyphyletic* populations are described as a group whose most recent common ancestor was not a member of the group (Ashlock 1971). Farris (in Wiley 1981) described polyphyly as a

group in which the most recent common ancestor was assigned to some other group and not to the group itself, or a group whose members have characters which are not uniquely derived. This includes characters are derived from parallel or convergent evolution.

The best defined subspecies are monophyletic, however, immigration by a few individuals from neighboring populations should not diminish the subspecific status of a population (O'Brien and Mayr 1990). Describing the phylogenies of populations, therefore, has ramifications for the management and protection of all animals. The result of removing the subspecific status from wood bison would raise questions about other endangered subspecies and their conservation programs. O'Brien and Mayr (1991) stated the conservation of a species or subspecies depends on maintaining the most genetically diverse populations possible, and by legislating protection for a species, subspecies or population on the grounds of a predetermined amount of genetic variability, we risk restricting the diversity and survival of our wildlife.

1.4 Objectives

The present study was performed to gather genetic information on bison and wapiti that would be useful to both taxonomists and wildlife managers, who are responsible for decisions affecting the survival of a species, who are interested in the interactions and movements of populations, and who must make restocking decisions.

The application of PCR technology made a survey of large populations of bison and wapiti possible. The d-loop region of mtDNA of both bison and wapiti was assessed for homogeneity, which in turn would indicate if the lack of variation between populations was typical of large North American mammals. The genetic variation of this region was also used to characterize both intra- and inter-populational differences. PCR products were restricted with enzymes, polymorphisms were noted and

haplotypes identified in each population. Sequence information allowed the confirmation of bison haplotypes with primers designed to assay specific sites. Differences between populations and subspecies were accounted for by the number of haplotypes present and the number of differences between haplotypes.

Bison haplotypes were also used to construct a phylogeny to test the subspecific status of wood and plains bison. The subspecific status of wapiti was tested from the presence or absence of unique haplotypes. The phylogenetic tree inferred for bison, from restriction data, was confirmed with sequence data.

Chapter 2

Review of Bison and Wapiti in North America

This chapter outlines the origin, classification, historic distribution, and appearance of North American bison and wapiti subspecies. Each section concludes with an estimate of the population size when the first measures of preservation were taken to establish our present day populations.

2.0 Bison

Bison first appeared in southern and eastern Asia about 2.5 million Y.B.P. (McHugh 1972), and later moved northward across Siberia into North America (Guthrie 1966). Two species of bison that became established on the Bering land bridge were *B. priscus* and *B. antiquus* whereby the former is suggested to have given rise to *B. occidentalis* (Pielou 1991). Present day North American bison are likely a hybrid of *B. antiquus*, which moved southward through the ice-free Rocky Mountain corridor, and *B. occidentalis*, which remained in the north (Guthrie 1970; Harington pers. comm. 1993). Modern bison probably originated only 5000 years ago (Wilson 1969; Van Zyll de Jong 1991), which could explain the north-south split between bison subspecies if wood bison (*B. bison athabascae*) consisted of a larger proportion or only *B. occidentalis*, and plains bison (*B. b. bison*) consisted of mostly *antiquus* animals.

North American bison are formally classified into two subspecies: wood bison (*B. b. athabascae* Rhoads 1898) and plains bison (*B. b. bison*). Prior to this, North American bison were described as *Bos bison* by Linnaeus in 1758, as *Bison americanus* by Allen in 1876, and as *Bison bison* by Osborn in 1910 (Roe 1970; Geist

1991). Based on historical illustrations and photographs, Heck (1936) and Krumbiegel (1980) suggested the plains bison consisted of at least two distinct geographic forms: the southern plains bison (*B. b. bison*) and northern plains bison (*B. b. montanae*). This classification, however, was disputed by Van Zyll de Jong (1986). Recognition for the distinct northern bison form was based on second-hand information given by Allen (1876), Rhoads (1897), and McDonald (1981). Acceptance of wood bison as a subspecies was refuted by Hornaday (1889) and Garretson (1938), but received renewed support from an extensive morphometric study by Van Zyll de Jong (1986).

The North American bison ranges covered a vast part of the continent (Figure 2.0). The plains bison range covered an area from the southern Canadian prairies to the Atlantic coast, and as far south as Mexico (Roe 1951; Dary 1974). Wood bison were distributed in the northern Canadian prairies, North West Territories, Yukon, and Alaska, and possibly along the west coast. The reference to a wood bison type in northern Saskatchewan and Manitoba (Roe 1970) is not supported by morphometric evidence from specimens (Van Zyll de Jong 1986).

The first records of bison in their native habitat were given by the Spanish, and are not necessarily valued for their observation of the bison's physical appearance, but for their description of populations before the loss of habitat, hunting, or both. In 1521, Cortez (in McHugh 1972: 41) described the first bison he saw:

> "...with crooked Shoulders, with a Bunch on its Back like a camel, its Flanks dry, its tail large, and its Neck covered with Hair of a Lyon. It is cloven-footed, its Head armed like that of a Bull which it resembles in Fierceness and with no less Strength and agility..."

Later observations by Henry Youle Hind in 1860 and Ernest Thompson Seton in 1885 provided improved descriptions of the wood and plains bison:

> "The skin of the so-called wood buffalo is much larger than that of the common animal, the hair is very short, mane or hair about the neck short and soft, and altogether destitute of curl, which is the common feature in the hair of the prairie animal." (Hind in Rhoads 1897: 494)
> "..The plains buffalo are not always of the dark and rich bright brown which forms their characteristic colour. They are sometimes seen from white to almost black." (Hind in Roe 1970: 35) "...the colour [of wood bison] ranging from light brown to darker brown, becoming almost black on head, legs and belly."(Seton in Roe 1970: 29)

Even behavioral differences were noted between the two bison subspecies. Sir John Richardson in 1829 remarked, "the bison which frequent the woody parts of the country form smaller herds than those which roam over the plains, but are said to be individually of greater size" (Roe 1970: 35). The skittish nature of wood bison and their use of muskeg and forested habitats also made these individuals more difficult to hunt than plains bison, a combination that probably contributed to the survival of wood bison.

The most accurate descriptions of North American bison, however, were made after the great herds disappeared. An illustration of wood and plains bison is given in Figure 2.1. An adult male wood bison weighs up to 1300 kg, stands 182 cm at the shoulder, has darker and woollier fur, and a larger, broader skull than a plains bison (McHugh 1972; Ying and Peden 1977). A plains bison bull can weigh up to 1000 kg, stand up to 165 cm at the shoulder, and have a total length of 315 cm, including a tail of approximately 45 cm. The horn cores are larger at the base and closer to the head in wood bison (Van Camp 1989). Horns of the plains bison may have a spread of up to 90 cm at their greatest point (McHugh 1972). The procumbent forelock, the thinner and shorter facial hairs, the indistinct gradation of cape hairs to the hind quarters, and the angular blocked hump peaking in front of the shoulder are all indicative of the wood bison (Van Camp 1989). Plains bison have a more pronounced bonnet, cape, and chaps and a gently sloping back (McHugh 1972).

The survival of plains bison has relied almost entirely on their integration into ranches, where they prospered until their introduction into North American parks. Records of bison populations are scarce, largely because early settlers believed the bison herds could not be decimated, and people could not foresee their incredible impact on the bison populations. On January 1, 1889 Hornaday estimated the bison population had been reduced to 1,090 animals, with 550 bison located in the Northwest Territories, Canada; 200 bison in Yellowstone National Park; and 340 bison divided among wild herds and private ranches (Jenning and Hebbing 1983).

2.1 Wapiti

The subfamily Cervinae first appeared in the late Miocene and early Pliocene (*circa* 13 million Y.B.P.; Geist 1971). During the Pleistocene, ancestral red deer dispersed eastward into Siberia and westward into Europe; one group invaded northern Africa (Murie 1951; Geist 1971). The wapiti in Siberia crossed the Bering land bridge, entered North America, and became centrally distributed in the continent by migrating along the ice-free Pacific and Rocky Mountain refugia.

Presently there are four extant and two extinct subspecies of wapiti recognized in North America. Linnaeus gave the name *Cervus elaphus* to the North American wapiti in 1758. By describing the Eastern wapiti (*C. e. canadensis*), Erxleben in 1777 identified the first wapiti subspecies (Bryant and Maser 1982). The Eastern wapiti was raised to full specific rank (*C. canadensis*) by Borowski in 1780 (Bryant and Maser 1982). The Rocky Mountain wapiti (*C. e. nelsoni* Bailey 1935), Manitoban wapiti (*C. e. manitobensis* Millais 1915), and Roosevelt wapiti (*C. e. roosevelti* Merriam 1897) together have also been classified as *C. e. canadensis*, a subspecies of the European red deer, because they resembled each other, as well as the wapiti in the

forest belt of Asia (Flerov 1952, Geist 1990). The historical ranges inferred for the North American wapiti subspecies are depicted in Figure 2.2. Only extant Canadian wapiti were included in this study; therefore, the ranges for the Tule wapiti (*C. e. nannodes* Merriam 1905) and extinct Eastern wapiti (*C. e. canadensis* Erxleben 1777) and Merriam wapiti (*C. e. merriami* Nelson 1902) are given for purely illustrative purposes.

Wapiti that progressed southeastward became established in one of the four ranges described by Bryant and Maser (1982). The Rocky Mountain wapiti range extended across the northern Canadian prairies and south along the Rocky Mountains. The Manitoban wapiti range covered the region of the prairies best known as the Great Plains. The Eastern wapiti range ran parallel to the Manitoban wapiti range and the Atlantic coast, with its northern limit set by the Great Lakes and a southern limit north of Florida. The Merriam wapiti range occurred south of the Rocky Mountain wapiti range and covered the areas of Arizona, Texas, New Mexico, and Mexico. The Tule and Roosevelt wapiti ranges suggest they arose from a migration of wapiti along the ice- free western coast. The Roosevelt wapiti range extended along the west coast from southern British Columbia to southern California (Bryant and Maser 1982). The Tule wapiti range continued southward and was enclosed by the Sierra-Nevada-Cascade Mountains (Bryant and Maser 1982).

The early descriptions of wapiti by Europeans in North American served to identify the distributions of herds, but did little to distinguish one population from the next. This is indicated in the following accumulation of excerpts given in 1737 by Brickell (in Bryant and Maser 1982: 50):

> The Elk is a monstrous, large, strong and swift beast, in shape exactly like a Deer, but bigger than a Horse, and is reported to be fearful..."
> "They have two large Horns, which exceed in weight all Creatures that are yet known in the New World. Their Neck is short and thick, but the Ears and Back very long: Their Colour is like a Harts and

> sometimes all White..." "Their Horns generally weigh twelve or fourteen Pounds. It is reported that some of them are seventeen Hands high."

Unfortunately, most wapiti along the Atlantic coast were hunted to extinction before the subspecies could be described. However, as remaining wapiti populations were studied, slight differences in morphology were discovered (Table 2.0).

The establishment of a national park system in Canada and the United States was probably a turning point in the population decline of wapiti. The number of wapiti surviving in scattered herds varied throughout North America. No estimates were found for the number of wapiti living in the northern prairies or outlying regions of the Rocky Mountains. However, in 1904, only 55 wapiti were located in the Beaver Hill area of central Alberta, and this was thought to be the largest existing herd in Canada (Blyth and Hudson 1987). By 1907, when Elk Island National Park was established in the area, only 20 Manitoban wapiti were found. These animals have continued to increase at an average of 19 per cent per year with an average herd of 500 animals maintained first by sale and slaughter and more recently by using animals for restocking elsewhere (Blyth and Hudson 1987). Alternatively, the Rocky Mountain wapiti in Yellowstone National Park began with 15,000 - 40,000 animals in 1886 - 1910, and have been monitored between 5,000 - 10,000 in recent years (Murie 1979). Wapiti from these two parks have been used to stock parks within and east of the Rocky Mountains. Along the west coast, the early establishment of the Olympic National Park and Strathcona Provincial Park provided a home for Roosevelt wapiti, whereas the establishment of two Manitoban parks, Riding Mountain National Park (RMNP) and Duck Mountain Provincial Park (DMPP), which was stocked with elk from RMNP, provided a home for two populations of Manitoban wapiti.

Table 2.0. Summary of phenotypic characters of three subspecies of wapiti; Rocky Mountain (*C. e. nelsoni*), Manitoban (*C. e. manitobensis*), and Roosevelt (*C. e. roosevelti*).

Character	Rocky Mountain	Manitoban	Roosevelt
Coat color	light reddish brown, neck, and legs brown	darker chestnut brown head and neck, rump straw color	dark brown head and neck in sharp contrast with paler body
Weight (kg)			
M	281 - 373	352	254 - 496
F	231 - 292	275	215
Total Body Length (cm)			
M	201 - 251	241 - 279	249
F	208 - 248	224 - 246	234
Tail Length (cm)			
M	13 - 16	12 - 17	8 - 11
F	12	13	10
Hind foot Length (cm)			
M	64.1 - 69.2	63.5 - 69.3	66 - 71.1
F	60.3 - 68.3	60.9 - 66.8	67.3
Height at shoulder (cm)			
M	-	147 - 156	152
F	-	137	127
Ear Length (cm)	19 - 21	21.6	20.3- 21.6
Antler:			
Beam Length (cm)	-	122 - 145	90 - 127
Circumference	-	19 - 20	25 - 36
Appearance	light, spreading	light, spreading	crown-like
Branches	straight	straight	short, heavy

note: compiled from the following references: H. U. Green (1933), D. C. Quimbly and D. E. Johnson (1951), J. E. Schwartz and G. E. Michell (1945), and W. A. Troyer (1960).

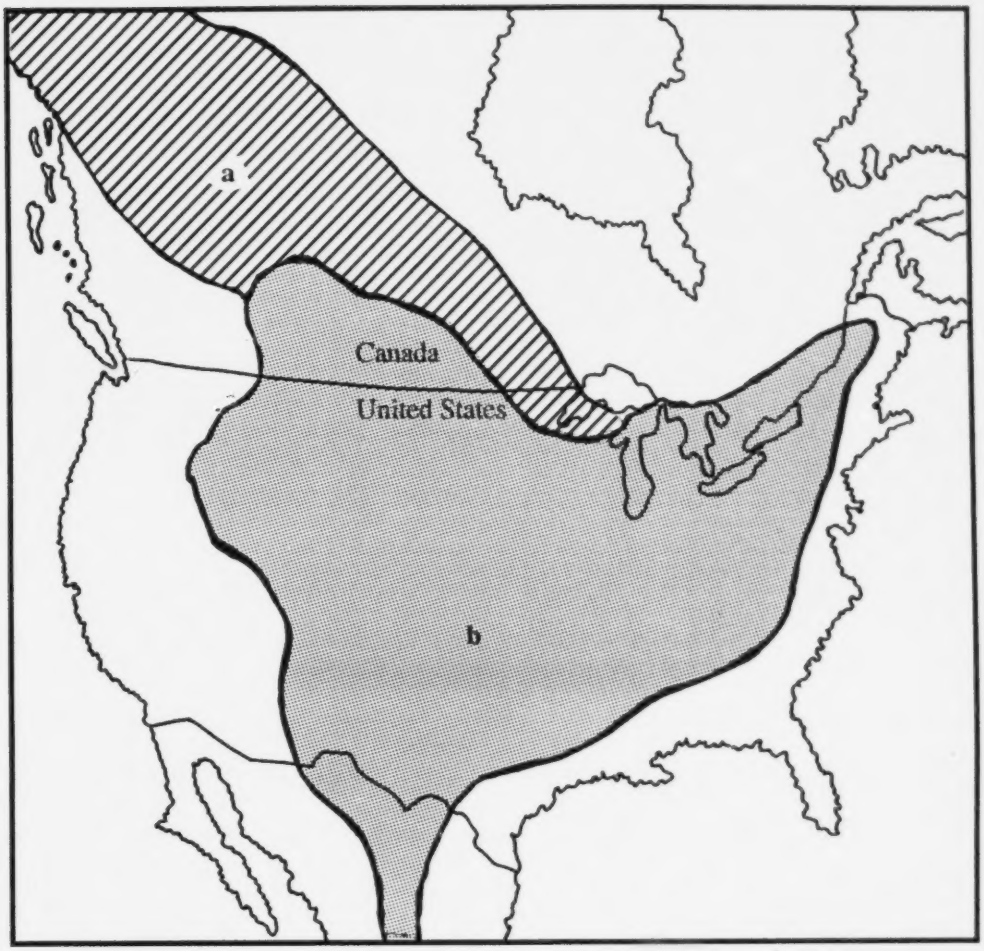
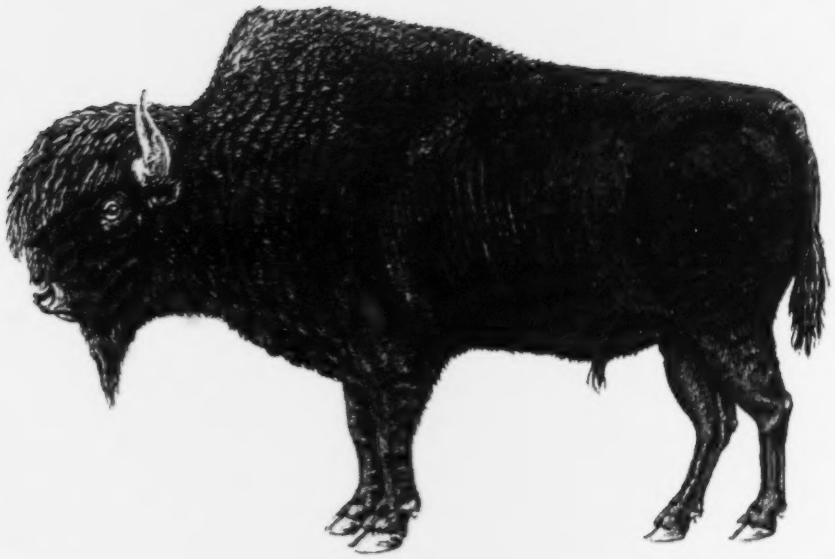


Figure 2.0 Historical distribution of North American (a) wood bison and (b) plains bison. Map adapted from McHugh (1972).



a) wood bison

b) plains bison

Figure 2.1 An illustration of a wood bison (a) and a plains bison (b) taken from Parks Canada 1987.

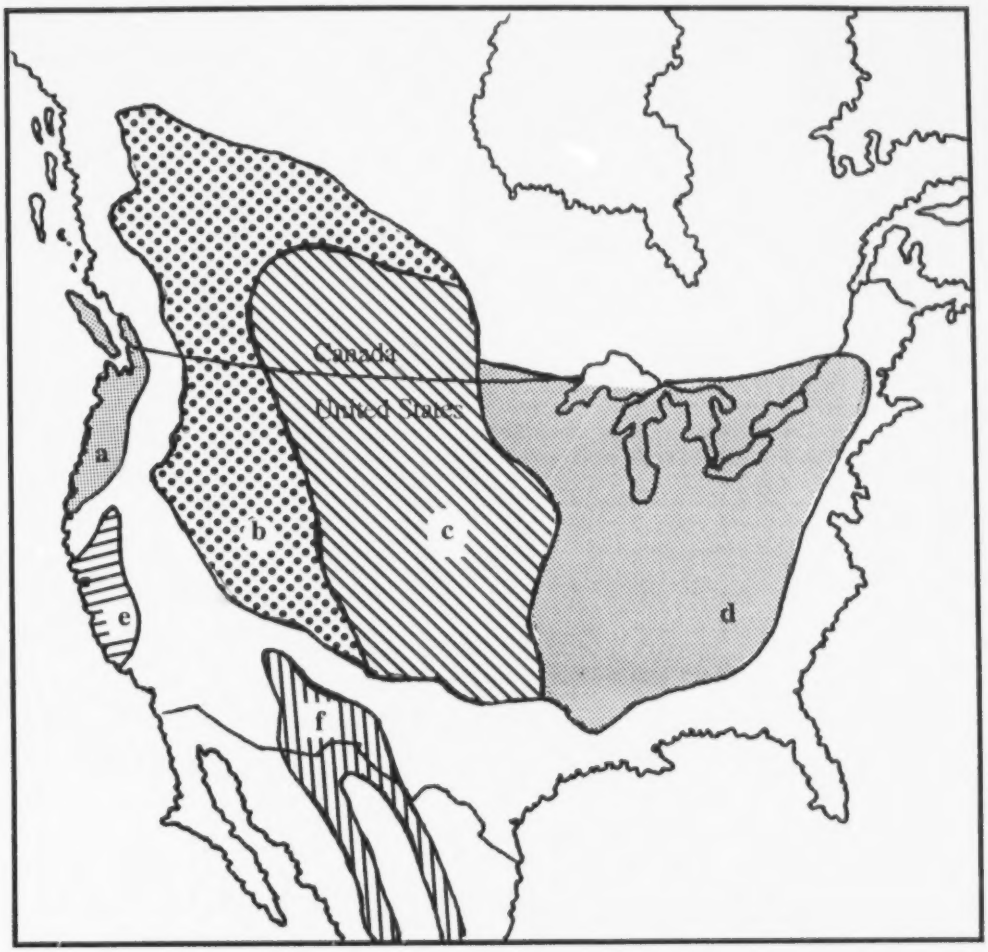


Figure 2.2 Historical distribution of North American wapiti where the ranges are indicated as follows: (a) Roosevelt wapiti, (b) Rocky Mountain wapiti, (c) Manitoban wapiti, (d) Eastern wapiti, (e) Tule wapiti, and (f) Merriam wapiti. Map adapted from Bryant and Maser (1982).

Chapter 3

Materials and Methods

3.0 Study Populations

Our ability to determine the historical diversity of bison and wapiti is limited to observations of early settlers, hunters, explorers, and natural historians. The reduced number of phenotypes and distribution observed today reflect in part the population bottlenecks wapiti and bison have both experienced. To estimate variation within and among extant populations, samples were obtained from several areas across North America. The populations chosen for this study included animals that would reflect the overall variation of the species.

This study focussed on wapiti presently found in Elk Island National Park, AB; Duck Mountain Provincial Park, MB; Banff National Park, AB; Rocky Mountain House, AB; Olympic Peninsula National Park, OR; and Strathcona Provincial Park, BC. Bison were sampled from the Mackenzie Bison Sanctuary, NWT; Wood Buffalo National Park, AB/NWT; Elk Island National Park, AB; Wichita Mountains Wildlife Refuge, OK; Custer State Park, SD; Ft. Niobrara Wildlife Refuge, NB; National Bison Range, MO; and Yellowstone National Park, WY.

While populations of wapiti are generally indigenous to their area, the bison populations in this study represent an accumulation of individuals from ten ranches. Therefore, the establishment of these founding herds is discussed first (section 3.0.1). The history of bison, beginning with their capture and integration into ranches, was

complicated by a turn-over of owners, trading of animals, and lack of records. This practice of trading and relocating bison continued even between parks, sanctuaries and zoos, and the relationship between these bison populations is outlined in section 3.0.2. Other herds involved in the preservation of bison are listen in Appendix A.

3.0.1 Captive Bison Herds

Alloway/McKay/Bedson/Strathcona In 1873 one male and two female bison calves from the Winnipeg area were captured for C.I. Alloway and J. McKay. The following spring another male and two female calves were captured from the Regina-Moosejaw area. Although one male calf died, by 1878 the herd had increased to fifteen (Ogilvie 1979). At that time eight animals were auctioned off to Colonel Sam Bedson of Stony Mountain, Manitoba (Dary 1974). Novakowski (1989) states Bedson also purchased an additional five bison and three cattalo (crosses between cattle and bison) from Alloway. Bedson borrowed money from Sir D.A. Smith (Lord Strathcona) to purchase the bison. Colonel Bedson captured three more calves and added them to his herd at Stony Mountain.

Wanting to improve his cattle herd, he crossed some of the bison with his domestic cattle (Ogilvie 1979) and in six years Bedson had a herd of 25 hybrids in addition to his 58 bison (Haines 1970). By 1887, Bedson had 127 bison (Bridges 1974). Twenty-seven of these were given to Lord Strathcona in repayment of his loan, and 100 animals, a third of which were cattalo, were sold to "Buffalo Jones" (Bridges 1974; Ogilvie 1979). Garretson (1938) however stated Bedson had only 80 bison and 13 cattalo, of which he sold only 86 animals (including cattalo) to Jones.

Jones shipped 30 bison from Manitoba to Kansas in December 1888 (Dary 1974). Although no records exist, the remaining herd must have been sent the following year. In 1898 Lord Strathcona gave 18 bison to the Canadian Government. Ogilvie (1979) stated 12 of these bison were shipped to Banff National Park and five remained in Winnipeg, while Garretson (1938) listed four bison as having stayed in Winnipeg, five slaughtered and the remaining nine sent to Banff.

Conrad C.E. Conrad of Kalispell, Montana bought 36 bison from Mrs. Allard in 1896 to supplement his herd of 27 (origin unknown). In 1902 when Conrad died, the herd consisted of 11 bulls, 23 cows and heifers, and 12 calves. The Conrad ranch continued operating, and in 1908, provided the original 37 bison to start the National Bison Range, in Montana. Between 1910 and 1911 he sold 30 bison to the Canadian Government, and these were later sent to Buffalo National Park at Wainwright, Alberta.

Corbin Austin Corbin, founder of the Blue Mt. Forest Park, purchased six male and six female calves from Jones (originally part of the Bedson herd) in 1888. In 1892 he bought another ten animals from Buffalo Jones; two bulls and eight cows which were of Texan origin (Garretson 1938). Novakowski (1989) stated 1882 as the date of purchase of the ten Jones bison. Conflicting dates often hinder efforts to accurately trace the origin of bison, especially when bison were traded among several ranches.

Dupree/Philip In 1881 Pete Dupree captured five calves near Fort Bennett, North Dakota. Although only three bison survived, by 1888 he had four bulls, five cows, and seven hybrids (Jenning and Hebbing 1983). The Dupree herd had grown to 83 bison when they were sold to James Philip. Philip had the animals moved to a pasture near Fort Pierre, South Dakota where they multiplied both naturally and through contributions made by ranchers who found bison scattered among their cattle herds during round-ups. Unlike most "buffalo men" of his day, Philip did not like buffalo hybrids, and said, "they were not worth a damn" (Dary 1974). Philip removed all hybrids from his herd. At the time of his death in 1911, the herd had peaked at over 400 animals and were sold to Custer State Park, W.R. Hurst, Miller Bros. 101 Ranch, W. Phillips, and H. O'Neil.

Eton Howard Eton purchased bison in 1896 from the Allard heirs (Garretson 1938). In 1902 these bison were sold to the United States government to establish a bison herd in Yellowstone National Park (Dary 1974).

Goodnight The Goodnight herd was established in 1878 from one male and one female calf from South Dakota. In 1879, three more calves were caught and two adult bulls (given as a gift to Mrs. Goodnight) were added to the herd (Garretson 1938). Although the Goodnight herd had only 13 bison in 1887 (Dary 1974), by 1933 the herd had grown to 250 animals (Garretson 1938). The Goodnight ranch near Palo Duro Canyon was also a source of cattalo. In 1899, Goodnight sold three bulls

and one cow to the New York Zoological Society and in 1902 he sold three bulls to Yellowstone National Park. One cow was shipped to the National Bison Range in 1909 to help establish a nucleus of bison in the park. In 1929 when Goodnight died, the Great Southern Life Insurance Company assumed maintenance of the animals.

Jones Between 1885 to 1889 Colonel "Buffalo" Jones caught 24 male and 33 female bison calves in Texas. He also bought ten adult animals from ranches in Kansas and Nebraska. He sold his first eight full grown calves from this herd to Austin Corbin in 1882 (Garretson 1938) or 1888 (Jenning and Hebbing 1983). In 1888 Jones purchased bison from Bedson. The number of bison reported to have been purchased ranges from 83 to 100 (Ogilvie 1979; Jenning and Hebbing 1983; Novakowsi 1989). From these animals he sold either ten or 12 bison to Corbin in 1888 (Novakowski 1989) or 1891 (Ogilvie 1979).

By the end of the summer of 1889, the Jones bison herd was suggested to number 140, including 56 Texas bison and their calves, plus 58 bison and some cattalo from the Bedson herd (Haines 1970). From time-to-time Jones sold off bison, including five pairs to England (Haines 1970). The number of bison he sold to Pablo-Allard cannnot be determined. Either he sold 67 animals to Pablo-Allard in 1893 and in 1895 bought them back (Dary 1974; Garretson 1938; Ogilvie 1979); or he sold 26 bison and 18 cattalo in 1895 (Haines 1970) and repurchased them in 1905 (Dary 1974).

Novakowski (1989) suggests Jones bought back some of the bison from Pablo and Allard, plus a few from Corbin, and established a herd in Arizona with bison and cattalo purchased from E. J. Molera of Monteray, California in 1904. In 1913, he and his partner divided the herd into equal shares and in 1915 sold his share to J. Owens, a private rancher. This marked the end of the Jones herd.

McCoy In 1882, Allen and Miner McCoy of Oklahoma caught two bison calves; one male and one female. The bison were given as gifts to the city of Keokuk, Iowa in 1884. Two years later the city gave two bison back to the McCoys, kept two, and sold two to the Page Woven Wire Company of Adrian, Michigan. On October 19, 1904 the New York Zoological Gardens purchased three cows and one bull from the Page Wire Fence Company (Dary 1974).

Pablo/Allard Dary (1974) and Olgilvie (1979) trace the origin of the Pablo-Allard herd to a Pend d'Oreille Flathead Indian, called Walking Coyote, who left Sweetgrass, Alberta in 1872 with eight calves. The following year, Walking Coyote arrived with six bison calves at his home on the Flathead Indian Reservation near Buffalo, Montana. Forced to leave the reservation, he again moved his calves until only four survivors remained; two males and two females (Garretson 1938; Aniskowicz 1990). By 1883 the bison herd had expanded to 14 (Ogilvie 1979) and in 1884, 12 were sold to Charles Allard and Michel Pablo (Ogilvie 1979; Aniskowicz 1990). Other reports insist ten or 14 bison were purchased (Dary 1974; Jenning and Hebbing 1983;

Novakowski 1989). By 1884, the herd had increased to 35 (Jenning and Hebbing 1983).

During the 1890's, Pablo and Allard purchased both bison and cattalo from Buffalo Jones, thereby adding to the collection of bison from Texas and Manitoba. Some of these animals were repurchased by Jones several years later.

When Allard died in 1896, 150 bison were divided among Allards' family and 150 were given to Michel Pablo. Shortly thereafter, Pablo sold his share to the Canadian Government (Aniskowicz 1990). Mrs. Allard sold her share of 36 Montana/Texas bison to Charles Conrad of Kalispell, Montana. The bison owned by their two daughters and one son were sold to Howard Eton. The bison of the second son were sold to Judge Woodrow of Missoula and then to the Miller Bros. 101 Ranch in Oklahoma.

The Government of Canada eventually purchased 716 bison from Michel Pablo (Ogilvie 1979; Parks Canada 1987; Novakowski 1989). The first shipments were sent to Elk Island National Park and acounted for the arrival of 199 bison June 1 and 211 bison October 22, 1907 (Federal Review 1990). In 1908, all bison were to be rounded up from EINP and shipped to Buffalo National Park, however, this proved impossible because either the bison were purposely not found or they were able to avoid capture. Shipments that followed went directly to BNP at Wainwright and included the arrival of 190 bison on July 3, 1909; 28 bison November 4, 1909; 28 bison June 21, 1910; and 28 bison October 19, 1910. The last seven bison that could be rounded-up in the Flathead country were shipped on June 16, 1912 (Dary 1974;

Ogilvie 1979), while the remaining 70 or so bison were eliminated by neighbours (Ogilvie 1979).

Whitney In 1897, W. C. Whitney purchased three bison bulls and ten bison cows from H.K. Glidden of Jackson, Wyoming and moved them to October Mountain, Massachusetts. He also bought a bull from Corbin that was originally caught in Texas by Buffalo Jones (Dary 1974; Ogilvie 1979). In 1901, Whitney shipped this bull to the New York Zoological Park. Two years lated he sent another 26 bison.

3.0.2. Present Bison Herds in North American Parks and Wildlife Refuges.

Park	Acronym	Location
Custer State Park	CSP	South Dakota
Elk Island National Park	EINP	Alberta
Fort Niobrara Wildlife Refuge	FNWR	Nebraska
Mackenzie Bison Sanctuary	MBS	Northwest Territories
National Bison Range	NBR	Montana
Wichita Mts. Wildlife Refuge	WMWR	Oklahoma
Wood Buffalo National Park	WBNP	NWT and Alberta
Yellowstone National Park	YNP	Wyoming and Montana

Custer State Park, South Dakota Custer State Park, established in 1913 on 24,848 hectares near Hermosa, South Dakota, was the last of the large American parks to be dedicated to the preservation of bison. In 1914 six bulls, 18 cows and 12 calves were purchased from Scotty Philip at Fort Pierre, SD (Dary 1974). Rorabacher (1970) states only 25 bison were purchased from Philip. In 1919 another 12,141 hectares were added to the park. In 1933, the population was near 300 and has been maintained at this level since then (Rorabacher 1970). In 1935 and 1937, two and

four bulls respectively, were shipped to Fort Niobrara, Nebraska. In 1951, 60 bison were donated to the park by the Sioux.

Elk Island National Park, Alberta Plains bison purchased in 1909 from Michel Pablo were shipped to and held at Elk Island National Park until Buffalo National Park at Wainwright was completed. At the time of transferring bison to Wainwright, about 50 bison were retained at EINP. By 1936, there were 2,479 bison in the park as a consequence of this action. Today, this population is closely monitored and is maintained at approximately 1,000 animals.

Wood bison have also been brought to the park, although these are kept in a confined area, separate from the plains herd. Of the 24 bison that were captured in Wood Buffalo National Park in 1965 (Federal Review 1990), 23 were safely brought to EINP in August (Aniskowicz 1990). One bison died shortly after arriving, which left five males and 17 females. The following year the first calf was born. In 1969, the original bison and the new bison born in the park were segregated; the original bison were destroyed to prevent an outbreak of tuberculosis from spreading. In 1969, the wood bison population in EINP was at 32 animals (Blyth and Hudson 1987) and today is maintained at about 500 animals.

On July 28, 1978 the first release of wood bison from EINP was made at Snake Indian Valley, Jasper National Park, Alberta. Two weeks later, the 21 animals had migrated to Grand Prairie and had to be shipped back to EINP (Ogilvie 1979). In 1980, the plains bison at BNP were replaced with wood bison from EINP.

Fort Niobrara National Wildlife Refuge, Nebraska The Fort Niobrara Wildlife Refuge near Valentine, Nebraska began with six bison donated by J.W. Gilbert of Nebraska (Garretson 1938). In 1913, two bison bulls were added from Yellowstone National Park (Rorabacher 1970) and $200 was raised by the local citizens to have the refuge fenced and the bison were released from their paddock (Garretson 1938). Between 1935 and 1937, six bulls were sent from CSP (Novakowski 1979). In 1942 four bulls were shipped to Wichita Mountains Wildlife Refuge, Oklahoma, and in 1952 two bulls were exchanged with the National Bison Range, Montana (Novakowski 1979).

National Bison Range, Montana In 1908, the Senate of the United States called for the establishment of a national bison range on the Flathead Indian Reservation in Montana. The American Bison Association arranged to provide the nucleus of animals for the NBR (Rorabacher 1970). The NBR established near Moiese, Montana recieved its first bison October 17, 1909. Twelve male and 22 female bison were shipped from the Conrad Estate at Kalispell, Montana, plus two bison as gifts from the Conrad Ranch and one cow from the Goodnight herd. The following year one bull and 12 cows were donated by the Blue Mt. Forest Association (Dary 1974) and an additional three from the Conrad herd (Novakowski 1989). In 1952, two bulls were shipped to FNWR, and a year later replaced with two bulls sent from Yellowstone National Park. In 1984, four cows were shipped to the park from Wichita Mountains Wildlife Refuge.

Mackenzie Bison Sanctuary, Northwest Territories A population of 200 pure wood bison was believed to have been observed in the Nyarling River region of Wood Buffalo National Park in 1959 (Novakowski 1989). In 1963, 18 of these bison were rounded up and sent to an area north of Fort Providence in the NWT, which later became known as the Mackenzie Bison Sanctuary (Roe 1970; McCormack 1992). Although only 16 bison survived the trip, the free-roaming population is now the largest herd in North America, with over 2,000 animals. This exponential growth shows no problem with inbreeding depression.

Wichita Mountain Wildlife Refuge, Oklahoma In 1901, the United States Congress opened 25,403 hectares of the Kiowa and Comanche Reservation in Cache, Oklahoma and in 1905 it was renamed the Wichita Mountain Wildlife Refuge. In 1906, the Wichita Forest and Game Reserve fenced off 3,238 hectares of the reservation for the bison. In 1907, under Hornaday's supervison, six male and nine female bison were chosen from the New York Zoological Gardens (Garretson 1938; Dary 1974). These bison arrived at the WMWR on October 17, 1907 (Dary 1974).

Wood Buffalo National Park, Alberta and NWT In 1893, fewer than 300 bison from the Great Slave Lake area were placed under the protection of the Northwest Mounted Police (Dary 1974). With this protection, the population grew such that when the park was given its official name in 1922, the population numbered approximately 1,500. Between 1925 and 1928 an estimated 6,673 bison were shipped from

Wainwright to WBNP (Fed. Rev. 1990). Because the population dramatically increased over the next few years, the park's boundary was extended in 1930.

In 1957, a discovery was made of what was believed to be the last pure wood bison in WBNP (Parks Canada 1987). Novakowski (1989), however, stated that in 1959 200 pure wood bison were sighted. Five of these were shot and sent to be identified in Ottawa (Dary 1974). In 1963, 18 bison were sent from WBNP to the MBS (Fed. Rev. 1990) although only 16 arrived safely (Aniskowicz 1990). In February 1965, 69 wood bison were found near Needle Lake. Forty-seven of the herd were captured and in 1966, 23 were shipped to EINP. Tests for tuberculosis showed the "pure wood" bison may not have been completely isolated (Ogilvie 1979).

Yellowstone National Park, Wyoming and Montana In 1872, when Yellowstone National Park was established, there were several hundred bison within its boundaries. Rorabacher (1970) claimed that these bison were not a separate species of bison (mountain bison), but rather plains bison that had used the park as a refuge. Support for Rorabacher is given by Dary (1974), who stated that the appearance of bison introduced at a later date to the park and bison captured from the indigenous herd was the same. During the winter of 1893 and 1894, poachers killed 120 bison that sought refuge in YNP. Twenty bison remained and these were finally put under full protection in 1894 (McHugh 1972). The population remained low until 1902 when additonal plains bison were introduced (Dary 1974).

In 1902, Buffalo Jones, warden of Yellowstone, set out to enlarge the herd in the park. He purchased 18 bison cows from Howard Eton (Pablo-Allard origin) and three bulls from Charles Goodnight of Goodnight, Texas (Rorabacher 1970). When one bull died that winter, the remaining animals were placed in captivity. Additions to the captive herd was made when three calves (two male and one female) were captured from the wild population. Possibly one or two more bison were added at a later date (Garretson 1938). Until 1968, the park maintained the population at approximately 500 animals. However, since then they have allowed the number of bison to fluctuate with the park's carrying capacity (Rorabacher 1970).

Table 3.0 A summary of bison populations found in North American parks, refuges and zoos. The superscripts cross reference parks and populations sharing bison from the same origin.

Park	Source	Origin
CSP[1]	Philip	North Dakota/Saskatchewan/Manitoba
	Conrad	Pablo-Allard[8]
EINP[2]	Pablo-Allard[8]	Montana/Alberta and Jones[11]
	Wood Buffalo[6]	
MBS	Wood Buffalo[6]	
NBR[3]	Goodnight[9]	South Dakota
	Corbin[10]	Jones[11]
	Custer[1]	
	Niobrara[4]	
	Wichita[5]	
FNWR[4]	Yellowstone[7]	
	Gilbert	Nebraska
	Custer[1]	
	National Bison Range[3]	
WMWR[5]	New York Zoological	Page Wire Fence Co.
		McCoy: Oklahoma
		Goodnight[9] and Hewans: Oklahoma
	Whitney	Corbin[10] and Glidden: Wyoming
	Corbin[10]	Jones[11]: Texas/Kansas and Allard[8]
		: Bedson: Saskatchewan/Manitoba
WBNP[6]	indigenous	
	Buffalo NP	Elk Island[2]
		Pablo Allard[11]
		Banff[1] and Boyd: Ontario
YNP[7]	Eton	Pablo-Allard[11]
	Goodnight[9]	
	indigenous	
	Jones[11]	

3. 1 Methods

A step-by-step outline, from collection to analysis, is given for the bison study. Following the bison section is a brief overview of the procedures and analysis applied in the wapiti survey.

Bison

3.1.1 Sample Collection

Blood was collected from the tail vein of 50 wood and 50 plains bison at Elk Island National Park. About 7 ml of blood was collected in ethylene diaminetetraacetate (EDTA) vacutainers (Becton Dickinson and Co., Rutherford N.J.), gently shaken, and immediately stored on wet ice until extraction. Approximately 35 ml of blood was collected from each of 58 bison at Wood Buffalo National Park. Samples were taken from four areas of the park (Appendix B), including: 21 from Sweetgrass, 21 from Pine Lake, 8 from Garden Creek, and 8 from Little Buffalo. These samples were stored and shipped on ice until processing. Twenty-two blood samples were collected in EDTA vaccutainers from bison at the Mackenzie Bison Sanctuary and shipped on ice by C. Gates. Blood samples also collected in EDTA or heparinized vaccutainers were provided by the United States Fish and Wildlife Service from the following areas: 12 from Yellowstone National Park, 22 from National Bison Range, 20 from Fort Niobrara Wildlife Refuge, 20 from Wichita Mts. Wildlife Reserve, and 30 from Custer State Park. Samples were shipped on ice and stored at 4 ^{o}C until processing.

3.1.2 DNA Preparation From Whole Blood

DNA was isolated from whole blood by first isolating and then lysing the white blood cells. To remove the red blood cells and serum, the blood was transferred to a 30 ml Corex® tube and an equal volume of 0.15 M ammonium chloride, 0.01 M potassium bicarbonate, 0.1 mM disodium EDTA (ACK) at 0 ^{o}C was added. The sample was gently shaken for 2 minutes and then centrifuged (Beckman model J2-21 M/E JA 20 rotor) at 2000 - 2500 rpm for 5 minutes. The supernatant was removed by aspiration and 15 ml of ACK was added. The step was repeated until the pellet was reasonably white. The pellet was washed in 15 ml of PBS (15.0 mM sodium phosphate dibasic, 0.137 M sodium chloride, 1.5 mM potassium phosphate monobasic and 2.7 mM potassium chloride) and centrifuged at 2000 rpm for 5 minutes. Again the supernatant was removed by aspiration and the pellet resuspended in 5 ml of PBS. Cells were lysed with the addition of 100 µl 0.5 M EDTA, 250 µl 20 % SDS (sodium dodecylsulphate), 10 ml TE pH 8.0 (10 mM Tris.Cl/1 mM EDTA) and 1.0 mg Proteinase K (Sigma Co., St. Louis MO). The purpose of lysing is to break open cells while leaving the organelles intact. The solution was agitated for several hours and left to incubate at 54 ^{o}C overnight.

3.1.3 Purification

In a series of extractions, nucleic acids were purified from the lysed cell solutions containing lipids, proteins, and other organic contaminants (Sambrook *et al.* 1989). The first extraction required adding an equal volume of Tris (50 mM Tris HCl pH 8.0) - saturated phenol and mixing for 10 minutes. The emulsion was centrifuged at 5000 rpm for 10 minutes. The upper aqueous layer was collected and re-extracted

with an equal volume of Tris-saturated phenol. Again the aqueous layer was removed and further extracted with an equal volume of 1:1 phenol/chloroform:isoamylalcohol. The solutions were mixed and centrifuged at 5000 rpm for 10 minutes. The final extraction of impurities from the aqueous layer used an equal volume of 24:1 chloroform/isoamylalcohol which served to remove traces of phenol that can cause degradation of DNA. The final aqueous phase was precipitated using 1/3 volume of 7 M ammonium acetate and an equal volume of isopropanol, thoroughly mixed, and centrifuged at 4 ^{o}C at 10,000 rpm for 1 hour. Finally, the DNA sample was resuspended in 2 ml of 1 x TE pH 7.0.

3.1.4 Amplification of the Displacement Loop (d-loop)

The d-loop region of mitochondrial DNA was enzymatically amplified using the polymerase chain reaction (PCR) in a final reaction volume of 100 µl solution containing 0.2 mM of each deoxynucleotide dATP, dTTP, dCTP and dGTP, 1 x *Thermus aquaticus* (*Taq*) magnesium-free polymerase buffer (Promega, Madison WI), 2.0 uM magnesium chloride, 20 pM of primers 1 or 39 and 2, genomic DNA (10 - 1000 ng), 1 unit of *Taq* polymerase (Promega, Madison WI) and brought up to volume with sterile double distilled water. Between 50 and 75 µl of mineral oil was added to each sample prior to amplification to prevent evaporation. Initial amplification cycles using the PHC-2 Techne Thermocycler followed guidelines set by Innis *et al.* (1990), and were as follows: denaturation at 94 ^{o}C for 4 minutes, annealing at 50 ^{o}C for 1 minute, extension at 72 ^{o}C for 1 minute, followed by thirty cycles of 94 ^{o}C at 1 minute, 50 ^{o}C at 1 minute, and 72 ^{o}C at 5 minutes, completed by 94 ^{o}C at 1 minute, 50 ^{o}C at 1 minute, and 72 ^{o}C at 10 minutes. Later these cycles were shortened to the following schedule with no change in yield or by-products: 94 ^{o}C at 5 minutes, 54 ^{o}C

at 30 seconds, 72 °C at 2 minutes, followed by 30 cycles at 94 °C at 15 seconds, 54 °C at 30 seconds, 72 °C at 2 minutes, ending with 94 °C at 15 seconds, 54 °C at 30 seconds, and 72 °C at 10 minutes. The annealing temperature was raised to 58 °C on the Perkin Elmer Cetus Thermal Cycler 480 with no identifiable change in yield or secondary products.

The primer sequences used for amplification were based on the universal d-loop primers described by Kocher *et al.* (1989), which were designed from the consensus sequence for human, cow (*Bos taurus*), and mouse (*Mus musculus*), but given a bias towards cow. Primers were originally prepared by the Department of Microbiology at the University of Alberta, and later by an Applied Biosystems 391 PCR-mate DNA synthesizer in our lab. The d-loop was first amplified by primers 1 (5' GGAAGGCTG GGACCAAACCT 3') and 2 (5' TAATATACTGGTCTTGTAAACC 3'), where primer 1 was later replaced by 39 (5' GGGTCGGAAGGCTGGGACCAAACC 3'). The sequence determined by Beech (unpublished) and given in Table 3.1, was used later to design internal and allele specific primers for assaying informative sites in bison.

3.1.5 Identification of Informative Sites

Informative sites were identified by (i) restricting the entire d-loop fragment with restriction endonucleases, (ii) restricting only selected regions of the d-loop, (iii) using allele-specific primers, and (iv) using primers that generated a restriction site for a specific allele. Primers designed to assay the d-loop are given in Table 3.2, while restriction enzymes employed to assay sites identified in bison (Figure 3.0) are included in Table 3.3. Also listed in Table 3.3 are: the primer combinations used to amplify fragments containing specific variants, primer product sizes, the variants assayed with restriction enzymes, and the respective fragment lengths of the restricted DNAs.

i) Identification of informative sites using restriction endonucleases to analyze the entire d-loop fragment

The 1100 base pair (bp) bison d-loop fragment was digested with nine commercially obtained restriction endonucleases (Boehringer Mannheim, Laval QB., Bethesda Research Laboratories, Burlington ON., Pharmacia, Dorval QB). These included the following four-base restriction endonucleases: *Alu I, Cfo I, Dde I, Hae III, Hinf I, Hpa II, Sau 3A,* and *Taq I* ; and the six-base restriction endonuclease, *Sty I*.

For each restriction digest, approximately 100-200 ng amplified DNA was digested with 2 units of enzyme and incubated at 37 ^{o}C overnight in a 1/10 dilution of buffer, except for *Taq I* , which was incubated at 72 ^{o}C. Approximately 50 µl of mineral oil was added to samples restricted with *Taq I* to prevent evaporation. Optimum digestion was achieved when buffer-salt concentrations recommended by the manufacturer were decreased from high to medium, medium to low, or low to zero. Decreasing the salt concentration compensated for salt residues already present in the amplified products. The digested DNAs were electrophoresed using a Biorad vertical gel apparatus through a 4 or 6% acrylamide 1x TBE (0.090 M Tris-borate/0.002 M EDTA: Sambrook *et al.* 1989) gel buffered in 0.5 x TBE at 25 mA per gel to a maximum of 300 volts for 2 - 2.5 hours. The acrylamide was polymerized using 10 µl of 10% ammonium persulphate and 1 µl Temed (tetraethylmethylene-diamine (BDH)) per ml acrylamide (95% acrylamide/ 5 % bis). Gels were transferred to a baking dish or plexi-glass tray, and stained for 30 minutes with ethidium bromide (2.0 µg/ml). The stained gels were transferred to plastic wrap and photographed using Polaroid type 667 coaterless film (Polaroid, Cambridge MA).

DNA fragments were observed for RFLPs and the lengths were estimated by comparison to a commercial 123 base pair ladder (BRL). The ladder was prepared by

adding 1.5 µg DNA to 20 µl sterile distilled water and 3 µl loading buffer (1% SDS, 0.1% bromophenol blue, 100 mM EDTA, 50% glycerol), then heating to 65 ^{o}C for 5 minutes before loading.

ii) Identification of informative sites using restriction enzymes to assay selected regions of the d-loop fragment

Shorter regions of the d-loop were amplified from bison DNA using internal primers. Digested products of shorter amplified regions are advantageous because separation, under the gel conditions employed, is greater between smaller fragments. In addition, the total number of fragments observed on a gel was reduced and positive identification of variants was more reliable. Variants were observed at sites 221, 289 and 455 using primers 2 and 25; and at sites 429 and 607 using primers 16 and 39 (see Table 3.3 for a description of the primers).

The 513 bp fragment amplified with primers 2 and 25 was restricted with either 2 units of *Ssp I* or *Bfa I* (New England Biolabs, Beverly MA) at 37 ^{o}C or *Mae I* at 45 ^{o}C. The *Ssp I* endonuclease recognized variants at sites 221 and 229 and the resulting fragments were separated on a 6% acrylamide TBE gel. The endonucleases *Mae I* and *Bfa I* both recognized the 455 site, but performed poorly, even after salt residues were removed by precipitating the DNA with 4:1 ethanol (95%)/ammonium acetate (7M) and sperimidine (Sigma Co., St. Louis MO), to a final concentration of 1 uM, was added to improve digestion. Therefore, allele-specific primers were designed to assay this site.

The 886 bp fragment amplified using primers 16 and 39 was digested with 2 units of either *Hinf I* or *Sty I* , and incubated at 37 ^{o}C overnight. Fragments were separated by electrophoresis on either a 6% acrylamide 1x TBE gel or a 1% 0.5 x TBE agarose gel (Sigma agarose type IIA). Again fragments were identified by staining with ethidium bromide.

iii) Identification of informative sites using allele-specific primers

Based on sequence data previously collected by Dr. R. Beech in our lab, primers were designed to detect informative sites in the bison d-loop not directly identified with the aid of restriction endonucleases. One such site is found at the 813 bp position where adenine (A) has replaced guanine (G) in some individuals (Figure 3.1). Following the examples of Berg *et al.* (1989) and Sommer *et al.* (1992), allele-specific primers were designed to amplify only the d-loop regions of individuals with the 'G' variant. Under conditions of low primer (2 µM) or magnesium (1 mM) concentrations, only the DNA of the specific variant should be amplified. Similar allele-specific primers were designed to detect the 'A' variant also under identical stringent conditions. By designing primers for both alleles, negative controls are created, because if no product is produced with primers for one allele then a product should appear for the alternative allele. If no products are produced, then the conditions may be too restrictive for amplification. The primers designed for the variants at 813 site were as follows:

Allele	Position (bp)	Primer Number and Sequence (5' -> 3')	
A	794-814	71	GAGCACCAGCATAATGGTAA
	796-813	82	GCACCAGCATAATGGAA
	827-812	86	CTATATGTGCATGTTT
G	832-813	70	ATTGACTATATGTGCATGCC

PCR reactions were performed in a final volume of 50 µl using the Perkin Elmer Cetus Thermal Cycler using the conditions described in methods section 3.1.3. Either primer 71 or 82 was paired with primer 1 (described in Table 3.3) and either primer 86 or 70 was paired with primer 2 (described in Table 3.3). Products of

amplification with primer sets 82/1, 86/1, and 70/2 occurred for both alleles even under the most stringent conditions and often included secondary products. The primer combination of 71 and 1, used to assay the 'A' allele, gave the most consistent results. Generally, amplification of only the 'A' allele occurred under conditions of either low primer or low magnesium concentrations. However, occasionally PCR products were produced under only one these conditions. This suggested one of the following: 1) the individual had the G allele, 2) the concentration of DNA was not adequate to produce a product, or 3) the conditions were too restrictive for amplification of the sample. Consequently, a primer generated restriction fragment length polymorphism was designed to assay site 813.

iv) Identification of informative sites using primer-generated restriction fragment length polymorphisms (RFLPs) to verify alleles

Using information from the bison consensus sequence (Table 3.1), primers were designed to assay known informative sites. Previous attempts to assay the 813 site using allele specific primers under stringent conditions gave ambiguous results. Therefore, a primer was designed to verify the presence of the 'A' allele (see Table 3.3 for primer 102). Primer 102 generated a template-dependant restriction site for *Alu I* at the 5' end of the primer. Primer 24 completed the primer pair and annealed to the 3' end. Because primer 102 was designed to anneal to the complementary DNA strand, the DNA template was synthesized from the 839 site and completed at the 681 site (see Figure 3.1). The initial three bases (AGC) of the recognition site were included in the primer sequence, while the fourth base was determined by the DNA template. If the first nucleotide of the template was a 'T', the recognition sequence for *Alu I* would be completed, and the amplified DNA could be cleaved. Fragments resulting from

digestion of DNA amplified from the haplotype with the *Alu I* site at the 813 bp position would be 53, 41, 40, and 25 bp in length, as indicated in Figure 3.3(a). Any other nucleotide found at this position would result in the absence of the*Alu I* site. PCR amplification and subsequent digestion of DNA from haplotypes without this *Alu I* site would result in fragment lengths of 65, 53, and 41 bp as shown in Figure 3.3 (a). Although amplified samples that were cleaved had a 'T' at the 813 site, they were designated the complementary 'A' allele to correspond with the sequence given in Table 3.1. PCR products that were not cleaved were assigned the complementary 'G' allele.

To confirm the identity of the 'G' allele, another primer to survey the 813 site was designed. Primer 103, described in Table 3.2, was constructed such that a *Hae III* site would be recognized if the allele present was a 'G'. *Hae III* recognizes the sequence GGCC, and the first three nucleotides of this sequence were incorporated into the primer. When the 'A' allele was present, the template would continue to read GGCT, while the 'G' allele would continue to read GGCC (Figure 3.2). The fragments resulting from the *Hae III* digests of each allele were given in Figure 3.3 (b). Fragment lengths for the 'A' allele included 90 and 59 bp, while fragments for the 'G' allele included fragments 65, 59, and 25 bp.

DNA samples required for assaying the 813 site were amplified in 50 µl volumes with either primer pairs 102/ 24 or 103/ 24. The 158 base pair fragment was restricted at 37 ^{0}C with 2 units of *Alu I* or *Hae III* and the products electrophoresed on an 8% acrylamide gel at 25 mA for 1.5 - 2 hours. The gel was stained for 30 minutes in ethidium bromide.

The development of this technique opened the possibility to assay sites 364, 365, 427, 446, 455, 564, and 655. Primer pairs used to amplify d-loop regions containing these sites are listed in Table 3.2 and the resulting products described in Table 3.3. The nucleotides of the allele-specific primers which partially constitute the

restriction sites are highlighted. With the exception of primers used to assay variants at sites 355, 655, and 813, primers were not designed to anneal to the complementary strand of DNA. All samples were amplified in 50 µl volumes and restricted with 2 units of enzyme at 37 ^{o}C for 3 - 24 hours. Products of less than 250 bp were electrophoresed on an 8% TBE acrylamide vertical gel, while larger products were electrophoresed on a 6% TBE acrylamide gel. Gels were stained and photographed as previously described.

3.1.6 Phylogentic Analysis

The program MacClade® version 2.1 for Macintosh (Maddison and Maddison 1986) was used to analyze nucleotide changes and to construct a phylogenetic tree (Figure 3.0) for the bison haplotypes. Values from 1 to 8b were then assigned to each of the clades with respect to their inferred order of evolution. These values correspond to the groups identified using restriction enzymes or PCR generated RFLP's (Table 3.4) and are consistent with those trees created by PAUP (phylogenetic analysis using parsimony).

The nucleotide substitutions from each haplotype were analyzed for their phylogenetic content using the computer package PAUP 3.0 (Swofford 1991). The program is based on the principle of maximum parsimony, whereby construction of a tree requires the lowest number of evolutionary changes to explain the differences observed among variants. No constraints were placed on the trees. In other words, the trees were undirected, unrooted and did not use weighted characters. Both the heuristic and branch-and-bound approaches were used to search for trees. Heuristic trees are created through step-wise addition of branches followed by branch swapping. Branch-and-bound trees are created by fixing the start of each branch and then adding new

branches at alternative sites to each of the fixed branches. Consensus trees were made by condensing trees where branches occurred most often in the replicates, and applying both strict , semi- strict, and maximum-likelihood constraints on all branches. A consistency index for each character was calculated using PAUP 3.0.

The phylogenetic tree derived from the restriction data was used to illustrate the lineages of bison. Bison were examined for para- and polyphyly using Farris's (in Wiley 1981) method of deriving paraphyletic and polyphyletic groups, where paraphyly describes members in which a reversal of a character has occurred, and polyphyly describes members in which characters were not uniquely derived (parallel or convergent). To determine the classification of plains bison, all branches representing plains bison haplotypes were assigned a group membership number 1. Any branches representing the ancestral or out group and wood bison haplotypes were assigned values of 0. By working down the tree, branches with sister groups that share the same group number were assigned the same group number. If the sister groups have different group values, such as 0 and 1, the branch was assigned B. Once the membership number of the ancestral group was determined, branches scored with a B were reassigned with a 1 or 0 by moving back up the tree and basing membership numbers on the most recent common ancestor.

The classification of wood bison was also determined using this algorithmic method, except the wood bison groups were assigned the 1 and then scored along branches to identify reversals or convergent evolution. The out-group and any branches representing plains bison haplotypes were assigned 0.

3.1.7 Haplotype Frequency and Genetic Distance

Haplotypes were identified and frequencies were calculated for all bison populations. Populations were further analyzed for intra- (eq. 3.1) and inter- (eq. 3.2) population distances by adopting the equation for minimum genetic distance from Nei (1987):

$$(3.1) \qquad D_{xx} = \sum_{i=1}^{n} \sum_{j=1}^{n} \frac{N_i N_j d_{ij}}{N_T^2}$$

$$(3.2) \qquad D_T = \sum_{i=1}^{n} \sum_{j=1}^{n} x_i y_j d_{ij}$$

$$(3.3) \qquad D_{xy} = D_T - \left[\frac{D_{xx} + D_{yy}}{2}\right]$$

where N_i and Nj are the number of individuals with each haplotype i and j; and d_{ij} is the difference between the haplotypes; N_T is the total number of individuals in the population; D_T is the total distance between all haplotypes in populations X and Y; x_i andy_j are the frequencies of each haplotype; $(D_{xx} + D_{yy})/2$ is the average distance within population X and Y; and D_{xy} is the minimum genetic distance between populations X and Y. A dendrogram was created by grouping least distant populations through a sequential clustering algorithm or UPGMA (unweighted pair-group method with arithmetic mean).

To achieve an unbiased estimate of divergence or measure of differentiation between populations or subspecies, probabilities were based on the restriction sites identified within the d-loop region by the eight original four-base restriction enzymes.

From this information, the probability that sequences X and Y share the same recognition sequence at a given site (S) can be determined as:

$$S = \frac{2m_{xy}}{m_x + m_y} \qquad (3.4)$$

where $m_x + m_y$ is the the number of restriction sites in DNA sequences X and Y, and m_{xy} are the number of shared restriction sites by the two sequences (Li and Graur 1991). The proportion of nucleotide differences (p) is estimated as:

$$p = 1 - S^{1/r} \qquad (3.5)$$

where r is the number of nucleotides in the recognition sequence. The number of substitutions per site between the two sequences is estimated from knowledge of p using:

$$K = -3/4 \ln (1-4/3\, p) \qquad (3.6)$$

where p is the proportion of different nucleotides between the two sequences as determined in equation 3.5 (Nei and Li 1979). Time of divergence (t) was finally calculated as:

$$t = K/2u \qquad (3.7)$$

where K is equal to the number of nucleotides in the recognition sequence, and u is the rate of nucleotide substitution (Li and Graur 1991). The values for u for the d-loop region are estimated at $2.0 - 0.08 \times 10^{-6}$ substitutions/bp/year (Brown *et al* . 1973).

3.2 Wapiti

3.2.0 Sample Collection

Blood was drawn from 11 wapiti at Elk Island National Park, Alberta, and 4 wapiti at Duck Mountain Provincial Park, Manitoba. Approximately 7 ml blood was collected in EDTA vacutainers, shaken gently, and immediately stored on wet ice. Samples were stored at 4 ^{o}C until DNA extraction.

Tissue samples were also collected from carcasses by Alberta Fish and Wildlife and Parks Canada. Subsamples of approximately one gram of muscle tissue were taken from wapiti in the following regions: 11 at Banff National Park, Alberta, 4 from Rocky Mountain House, Alberta, 8 from Strathcona Provincial Park, British Columbia, 8 from Olympic National Park, Oregon, 1 from Westlock, Alberta, 1 from Colorado, and 1 from Kootenay National Park, British Columbia.

3.2.1 Isolation of DNA From Blood and Tissue

DNA was isolated from either white blood cells or tissue. DNA was collected from white blood cells after lyzing and removing the red blood cells as described in 3.1.2. DNA was isolated from tissue samples by homogenizing approximately 1.0 g of frozen tissue in 5 ml of TE and homogenized for 3 x 30 seconds using a Brinkman pt 10/35 homogenizer set at speed level 3. The homogenate was increased to a volume of 15 ml with lysis buffer (100 ug/ml proteinase K, 0.5 M EDTA and 0.5 % N-lauroylsarcosine (Sigma Co., St. Louis MO)) and incubated at 54 ^{o}C overnight.

3.2.2 Purification and Amplification

DNA was purified as described in 3.1.3 and the d-loop was amplified as in methods 3.1.4. The primer pairs 1 and 39, used to amplify the bison d-loop, also produced single amplified products from wapiti DNA.

3.2.3 Identification of Informative Sites Using Restriction Endonucleases

The amplified DNA of wapiti was digested with eight commercially obtained restriction endonucleases (Boehringer, Mannheim, Laval, QB., Bethesda Research Laboratories, Burlington ON, Pharmacia, Dorval QB). These included the following four- base restriction endonucleases: *Alu I*, *Cfo I*, *Hae III*, *Rsa I*, *Sau 3A* and *Taq I* , and five- base restriction endonucleases: *Dde I* and *Hinf I*.

For each restriction digest, approximately 100-200 ng amplified DNA was digested with 2 units of enzyme and incubated at 37 ^{o}C overnight in a 1/10 dilution of buffer, with the exception of *Taq I*, which was incubated at 72 ^{o}C. Approximately 50 µl of mineral oil was added to samples restricted with *Taq I* to prevent evaporation. Again optimum results were achieved when buffer salt concentrations recommended by the manufacturer were decreased by from high to medium, medium to low, or low to zero. The digested DNA was electrophoresed and the resulting gels were stained and photographed as previously described in materials section 3.1.5.

3.2.4 Divergence Between Haplotypes

Divergence or the measure of differentiation between populations or subspecies was calculated from restriction-fragment patterns. First the expected proportion of shared DNA fragments between two sequences of DNA (F) was calculated:

$$(3.8) \qquad F = \frac{2m_{xy}}{m_x + m_y}$$

where m_x and m_y are the numbers of restriction fragments resulting from the digestion of haplotypes A and B, respectively, and m_{xy} is the number of fragments shared by the two haplotypes. Nei and Li (1979) used the expected proportion of shared fragments (F) to calculate the probability (G) that a restriction site has remained unchanged over time:

$$(3.9) \qquad G = [F(3 - 2G)]^{1/4}$$

From this equation, the substitutions per nucleotide (K) and time of divergence (t) can be estimated:

$$(3.10) \qquad K = -(2/r)\, ln\, (G)$$

$$(3.11) \qquad t = K/2u$$

where r is equal to the number of nucleotides in the recognition sequence, and u is the rate of nucleotide substitution (Li and Graur 1991).

Table 3.1: Bison d-Loop Sequence. Base substitutions for domestic cow (*Bos taurus*) sequence are shown below the bison (*Bison bison*) consensus sequence, bison variants are shown above the line, and deletions are indicated with a (-).

```
                                                                                                  50
      Bison  TAATATACTG  GTCTTGTAAA  CCAGAAAAGG  AGAGCAACTA  ACCTCCCTAA
      Cow    ..........  ..........  .....G....  ...A......  ..........

                                                                                                 110
GACTCAAGGA  AGAAACTACA  GTCTCACCGT  CAACCCCCAA  AGCTGAAGTT  CTATTTAAAC
..........  .......G..  ........A.  ..........  ..........  ..........

                                                                                                 170
TATTCCCTGA  ACGCTATTAA  TATAGTTCCA  TAAATGCAAA  GAGCCTCACC  AGTATTAAAT
..........  ..A.......  ..........  .....A....  ......T.T.  ..........

                  G                                                                 C       230
TTACTAAAAA  TTCCAATAAC  TCAACACAAA  CTTTGTACTC  TAACCAAATA  TTGCAAACAC
..T.C.....  .C........  ........G.  A....C..C.  ..........  ..A.......

                                                                                  G       A
CACTAGCTAA  CGTCACTCAC  CC---------CCAAA--ATG  CATTACC--CAAAC-GGGGGA-AT
..........  .A.A..A..G  ..ATACACAGA...C.GA...  A......TA.GC.A.....T....

          T                                                                                      340
ATACATAACA  TTAATGTAAT  AAAAACATAT  TATGTATATA  GTACATTAAA  TTATATGCCC
..........  ..........  ...G.....A  ..........  ..........  ..........

                              TT          A T                                                 400
CATGCATATA  AGCAAGTACT  TATCCTCTAT  TGACAGTACA  TAGTACATAA  AGTTATTAAT
..........  .........A  .GA.......  -AG.......  ..A......T  .A.....G.C

                                    C T                                 C
TGTACATAGC  ACATTATGTC  AAATCTACCC  TTGGCAACAT  GCATA-------TCCCT-
..........T ..........  ....TC.TT.  ...AT.GT..  ATC..TTATATA.T...T

    C                               C                                                          510
TCCATTAGAT  CACGAGCTTA  ATTACCATGC  CGCGTGAAAC  CAGCAACCCG  CTAGGCAGAG
A.........  ..........  ..........  ..........  ..........  .........-.

                                                                                       T    570
GATCCCTCTT  CTCGCTCCGG  GCCCATGAAC  CGTGGGGGTC  GCTATTTAAT  GAACTTTATC
..........  ..........  ......A...  ..........  .....CC...  ...T....C.

                                                      A                                       630
AGACATCTGG  TTCTTTCTTC  AGGGCCATCT  CACCTAGAAT  CGCCCATTCT  TTCCTCTTAA
..G.......  ..........  ..........  ..T...A..C  G.T.......  ..........

                                    A                                                         690
ATAAGACATC  TCGATGGACT  AATGGCTAAT  CAGCCCATGC  TCACACATAA  CTGTGCTGTC
..........  ..........  ..........  ..........  ..........  ..........

                                                                                              750
ATACATTTGG  TATTTTTTTA  TTTTGGGGGA  TGCTTGGACT  CAGCTATGGC  CGTCAAAGGC
..........  ..........  ..........  ..........  ..........  ..........
```

Table 3.1 (continued): Bison D-Loop Sequence. Base substitutions for domestic cow (*Bos taurus*) sequence are shown below the bison (*Bison bison*) consensus sequence, bison variants are shown above the line, and deletions are indicated with a (-).

```
                                                                      810
CCTGACCCGG  AGCATCTATT  GTAGCTGGAC  TTAACTGCAC  CTTGAGCACC  AGCATAATGG
..........  ..........  ..........  .........T  ..........  .........A

  G                                               -  T                C      870
TAAGCATGGA  -CATATAGTCA ATGGTTACAG  GACATAAACT  GTATTATATA  T-CCCCCCTC
..........  T....C.....  .....C....  ........T.  A.........  ........T..

                                                                      930
CATAAAAATT  CCCCC-TTAAA TATTTACCAC  TGCTTTTAAC  AGATTTTTCC  CTAGTTACCT
ATA....T..  .....C.....  ...C......  CA........  ...C......  ....A...T.

                                                                      990
ATTTAAATTT  TCCACACTTT  CAATACTCAA  ATTAGCACTC  CATATAAAGT  CAATATATAA
..........  .T...G....  ..........  T.........  ..A.C.....  ..........

                                                                     1050
ACGCAGGCCC  CCCCCCCCCG  TTGATGTAGC  TTAACCCAAA  GCAAGGCACT  GAAAATGCCT
..........  ..........  ..........  ..........  ..........
      ..........

                                                           1100
AGATGAGTCT  CCCAACTCCA  TAAACACATA  GGTTTGGTCC  CAGCCTTCCG
..........  ..........  ..........  ..........  .........T
```

Table 3.2 D-Loop primers used to assay variable sites in *Bison bison*. The underlined regions represent primers which have a template dependant restriction site.

Primer	Location (bp)	Sequence Primer (5' - 3')
1	1120-1101	GGAAGGCTGGGACCAAACCT
2	1-22	TAATATACTGGTCTTGTAAACC
16	218-240	ATATTGCAAACACCACTAGCTAAC
18	814-792	CTTATCATTATGCTGGTGCTCAAG
24	681-700	CTGTGCTGTCATACATTTGG
25	538-515	TCATGGGCCGGAGCGAGAAGAGG
29	483-504	CGTGAAACCAGCAACCCGCTAGGC
39	1104-1081	GGGTCGGAAGGCTGGGACCAAACC
84	595-572	GCCCTGAAGAAAGAACCAGATGTC
102	839-814	TGTAACCATTGACTATATGTGCAAGC
103	839-814	TGTAACCATTGACTATATGTGCAGGC
107	433-454	GGCAACATGCATATCCCTTCCT
138	396-426	TAATTGTACATAGCACATTATGTCAAATGT
139	415-445	ATGTCAAATCTACCCTTGGCAACATGCGTA
149	686-656	AGCACAGTTATGTGAGCATGGGCTGATTGG
150	533-563	CATGAACCGTGGGGGTCGCTATTTAATGAT
155	333-363	TATGCCCCATGCATATAAGCAAGTAAATAT
156	396-366	TAACTTTATGTACTATGTACTGTCACTCGA

Table 3.3 Primers and enzymes used to assay variable sites in the bison d-loop

Site Tested (bp)	Primer Pair (A/B)	Product Size (bp)	Enzyme	Sequence Recognized by Enzyme	Variant Tested	Size of Fragment (bp)
221; 289	2/25	538	*Ssp I*	AAT' ATT	C, C	538
					C, T	289, 249
					T, T	249, 219, 70
364	155/25	205	*Ssp. I*	AAT' ATT	C	205
					T	175, 30
365	16/156	178	*Xho I*	C' TCGAG	C	148, 30
					T	178
427	138/25	142	*Rsa I*	GT' AC	A	112, 23, 7
					T	135, 7
429	16/39	886	*Sty I*	C 'CTAGG	C	675, 211
					T	886
	2/39	1082			C	675, 429
					T	1082
	2/1	1098			C	669, 429
					T	1098
446	139/25	123	*Rsa I*	GT' AC	C	94, 29
					T	123
455	107/84	162	*Xba I*	T' CTAGA	C	141, 21
					T	162
564	150/18	281	*Sau 3A*	' GATC	C	252, 29
					T	281
607	16/39	886	*Hinf I*	G' ANTC	G	497, 389
					A	886
	2/1	1098			G	607, 513
					A	1098
655	29/149	203	*Hae III*	GG' CC	G	63, 61, 48, 31
					A	92,63, 48
813	24/102	158	*Alu I*	AG' CT	G	65, 53, 41
					A	53, 41, 40, 25
	24/103	158	*Hae III*	GG' CC	G	65, 59, 25
					A	90, 59

Table 3.4 Classification of *Bison bison* haplotypes based on variants found at sites 221, 289, 364, 365, 427, 429, 446, 455, 564, 607, 655, and 813 respectively.

Group				
	1	TCCCATTTCAGA	6a	TCTCACTCCGGA
	2	TCCCATTTCGGG	6b	TCCCACTCTGGA
	3	TCCCACTTCGGG	7a	TCCCACTTCAGA
	4	TCCCACTTCGGA	7b	TCCCACTTCAAA
	5	TTCCACTTCGGA	8a	CCCCACCTCAGA
	COW	TCCCATTTTAGA	8b	CCCTACCTCAGA

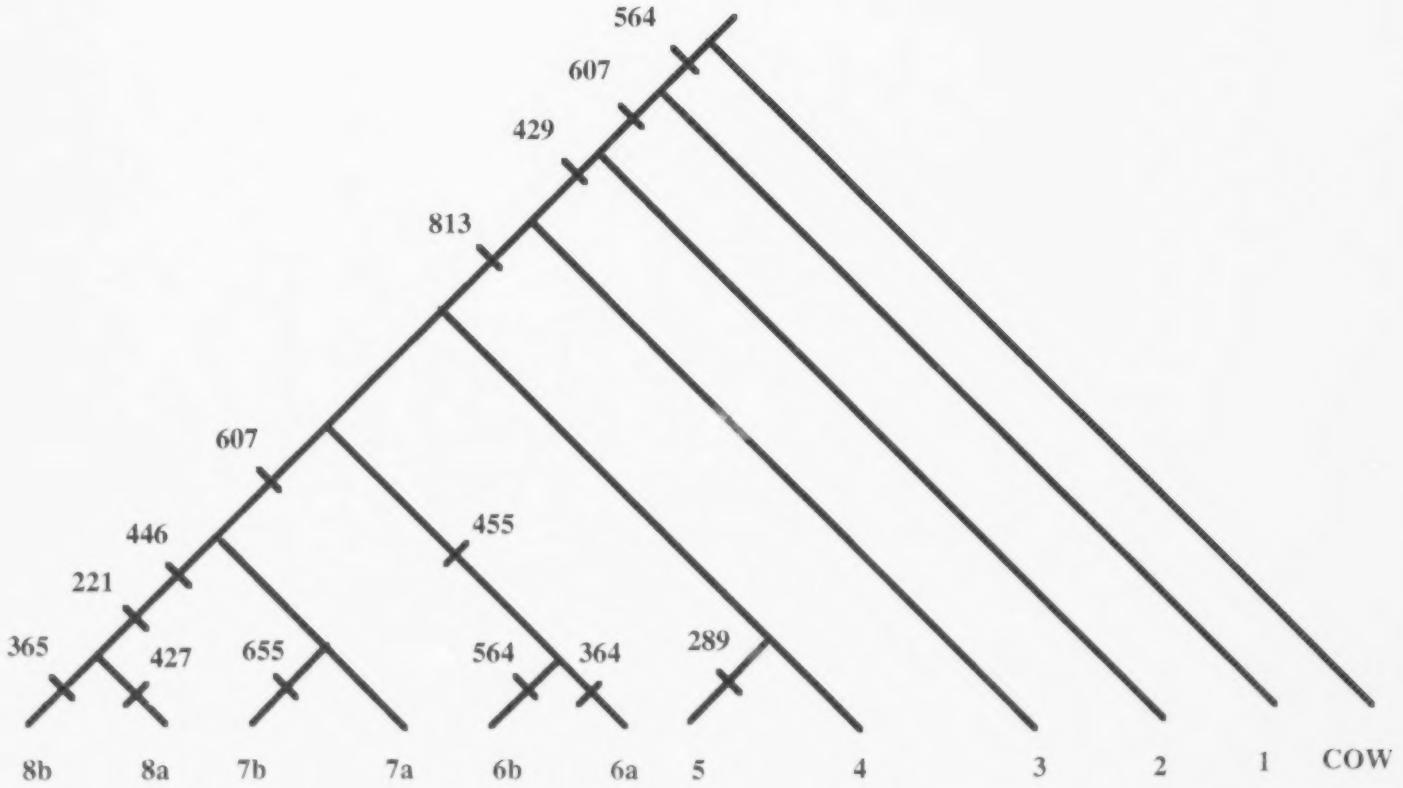
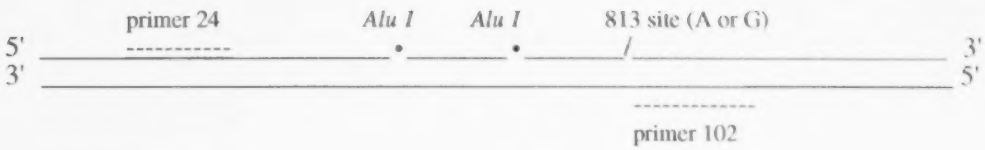


Figure 3.0 Phylogram of haplotypes 1 through 8b used to categorize individual bison. The phylogram is based on complete sequence data collected by Beech (unpublished 1990) and analyzed using MacClade™. These haplotypes are characterized by changes at nucleotide sites 221, 289, 364, 365, 427, 429, 446, 455, 564, 607, 655, and 813. The haplotypes described here are consistent with haplotypes described elsewhere.

Figure 3.1 Primer-generated restriction fragment length polymorphisms for the 'A' allele at 813 site in the bison d-loop. (a) The region of mtDNA, illustrated from position 1 to 1100, contains two restriction sites for *Alu I* and the annealing sites of primers 24 and 102. (b) The sequence of both alleles is given from sites 800 to 839, and the 813 site is marked with an asterisk. Primer 102 is shown annealed to the DNA. The nucleotide of the primer, shown in boldface, was selected to produce a template dependant restriction site. (c) Amplification for both alleles begins at position 839 and ends at 681 (not shown). The restriction sequence AGCT, contributed in part by the primer and completed by the template is underlined in the 'A' allele. This sequence was not recognized or restricted in the template where the restriction site was completed with a 'C' as in the 'G' allele.

a) d-loop



b) annealing of primers to d-loop

'A' allele

```
      800            *                                          839
5' - CAGCATAATGGTAAGCAGGACATATAGTCAATGGTTACAGGACTAAAC- 3'
                     CGACCTGTATATCAGTTACCAATGTCCTGATTTG
                                primer102
```

'G' allele

```
      800            *                                          839
5' - CAGCATAATGGTAGGCAGGACATATAGTCAATGGTTACAGGACTAAAC- 3'
                     CGACCTGTATATCAGTTACCAATGTCCTGATTTG
                                primer 102
```

c) PCR products of both alleles using primer 102

'A' allele

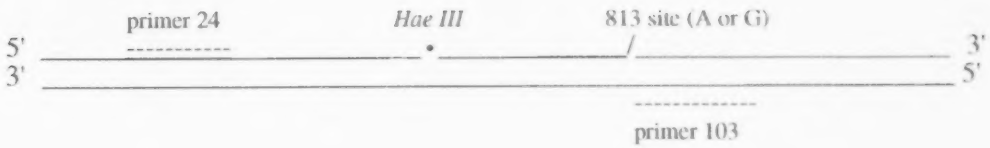
```
                       *                                        839
5'....CAGCATAATGGTAAGCTGGACATATAGTCAATGGTTACAGGACTAAAC 3'
3'...............<-TCGACCTGTATATCAGTTACCAATGTCCTGATTTG
                            primer 102
```

'G' allele

```
                       *                                        839
5'....CAGCATAATGGTAGGCTGGACATATAGTCAATGGTTACAGGACTAAAC 3'
3'...............<-CCGACCTGTATATCAGTTACCAATGTCCTGATTTG
                            primer 102
```

Figure 3.2 Primer-generating restriction fragment length polymorphisms for the 'G' allele at the 813 site in the bison d-loop. (a) The region of mtDNA, illustrated from position 1 to 1100, contains two restriction sites for *Alu I* and the annealing sites of primers 24 and 103. (b) The sequence of both alleles is given from sites 800 to 839, and the 813 site is marked with an asterisk. Primer 103 is shown annealed to the DNA. The nucleotide of the primer, shown in boldface, was selected to produce a template dependant restriction site. (c) Amplification for both alleles begins at position 839 and ends at 681 (not shown). The restriction sequence GGCC, contributed in part by the primer and completed by the template is underlined in the 'G allele. This sequence was not recognized or restricted in the template where the restriction site was completed with a 'T' as in the 'A' allele.

a) d-loop



b) annealing of primers to d-loop

'A' allele

```
     800              *                                                839
5' -CAGCATAATGGTAAGCAGGACATATAGTCAATGGTTACAGGACTAAAC - 3'
                    CGGCCTGTATATCAGTTACCAATGTCCTGATTTG
                                    primer 103
```

'G' allele

```
     800              *                                                839
5' -CAGCATAATGGTAGGCAGGACATATAGTCAATGGTTACAGGACTAAAC- 3'
                    CGGCCTGTATATCAGTTACCAATGTCCTGATTTG
                                    primer 103
```

c) PCR products of both alleles using primer 103

'A' allele

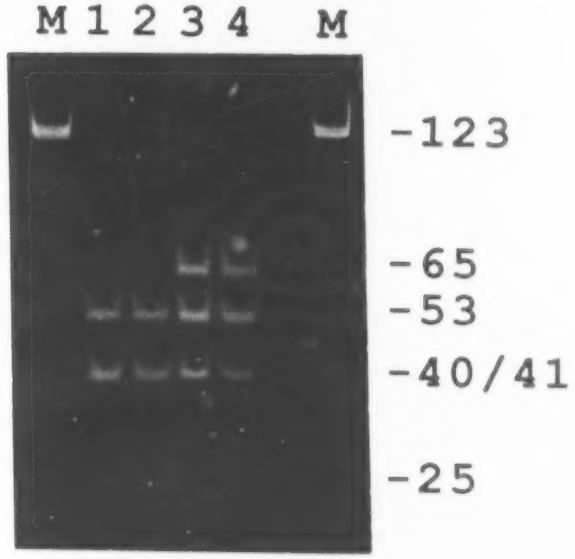
```
                        *                                            839
5'....CAGCATAATGGTAAGCCGGACATATAGTCAATGGTTACAGGACTAAAC 3'
3'................<-TCGGCCTGTATATCAGTTACCAATGTCCTGATTTG
                                primer 103
```

'G' allele

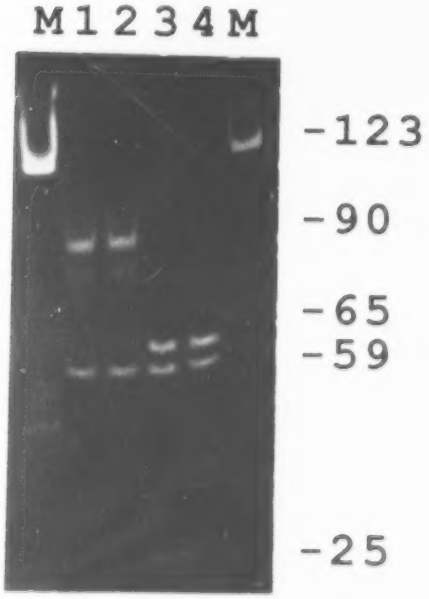
```
                        *                                            839
5'....CAGCATAATGGTAGGCCGGACATATAGTCAATGGTTACAGGACTAAAC 3'
3'................<-CCGGCCTGTATATCAGTTACCAATGTCCTGATTTG
                                primer 103
```

Figure 3.3 Identification of the restriction polymorphisms for both alleles at the 813 site in the bison d-loop using restriction enzymes *Alu I* and *Hae III*. The 'A' allele is identified in the *Alu I* digest by the presence of fragments 53, 41, 40 and 25 bp, whereas the 'G' allele is identifed by the presence of fragments 65, 53, and 41 bp (a). In the *Hae III* digest, the 'A' allele is identified by the presence of fragments 90 and 59 bp, whereas the 'G' allele is identified by the presence of fragments 65, 59, and 25 bp. The 123 bp marker is also observed.

(a) Alu I



(b) Hae III



Chapter 4

Results

4.1 Amplified d-Loop

For each of the 269 bison and 49 wapiti DNA samples extracted and amplified, a single double stranded product was visible after 30 or 40 cycles of PCR. The d-loop regions of mitochondrial DNA amplified from both the wapiti and bison species measured approximately 1100 base pairs in length. No inter- or intra-specific differences could be identified from whole amplified products.

4.2 d-Loop Haplotypes

DNA haplotypes were identified through the use of restriction endonucleases. Each restriction enzyme recognized and cleaved the DNA at a unique sequence of four, five, or six nucleotides. The resulting fragments were separated by size through gel electrophoresis, whereby small fragments migrated more quickly than large fragments. Each haplotype was represented by a specific set of restriction fragment length polymorphisms (RFLPs; Figure 4.0). Typical restriction digests gave unambiguous banding patterns. Within the three putative subspecies of wapiti and two putative subspecies of bison, three and 11 haplotypes, respectively, were found (Tables 4.0, 4.1, 4.2, and 4.3).

4.2.1 Bison

The eleven restriction enzymes applied to the entire bison d-loop region produced a total of 51 different fragments for the examination of intra- and inter-population variability. This included 22 fragments from primer-generated RFLPs (Table 3.2) and 29

fragments from direct restriction analysis (Table 4.4). The 29 fragments separated bison into four polymorphic groups; those with or without the 607 site (*Hinf I*) and/or the 429 site (*Sty I*), none of which separated plains or wood bison. By reducing the size of the amplified product to include only the region of interest, fragments that could complicate the restriction pattern were removed (Table 3.3). Variants at sites 221 and 289 were identified from a shorter region of the d-loop by the enzyme *Ssp I*. Only wood bison from Elk Island National Park (EINP) did not have individuals expressing the variant at site 221. The polymorphism at site 289, however, was present in only wood bison containing populations which are defined as EINP(wood), Mackenzie Bison Sanctuary (MBS) and Wood Buffalo National Park (WBNP).

Allele-specific primers were designed to look for variants at sites 364, 365, 427, 446, 455, 564, 655 and 813. The variants at sites 364, 455 and 564 were found in all bison originating from the founding population of WBNP, except MBS, which did not have the variant at site 564. The variant at site 365 was restricted to plains populations from Yellowstone National Park (YNP), National Bison Range (NBR), Custer State Park (CSP), and EINP. Site 427 should be polymorphic according to the sequence data of Beech (unpublished) but was found invariable in all populations. The variant at site 446, fixed in bison at the Ft. Niobrara Wildlife Refuge (FTWR), was shared by all other populations except the wood bison of EINP. The variant at site 655 was present in plains bison populations from NBR, CSP, and EINP; and in bison from WBNP. The variant at 813 site helped characterize haplotype 3, which was found in all populations except FNWR, and haplotype 2 which was only found in CSP.

The polymorphic sites within the d-loop region of 24 bison were given in Table 3.1 which also describes the consensus sequence (Beech unpublished). I assumed that the cleaved DNA would represent one of the two variants identified from the consensus sequence. In reality, the possibility exists that the enzyme did not restrict the DNA

because any one of three nucleotides found at this position, or additional changes in the recognition sequence, could cause the site to be unrecognized. As the number of changes in the d-loop region were few and no more than one variant was found at any such site, the likelihood of misclassifying a haplotype using this technique was low.

The genotype frequencies within different herds (see Table 4.0) exhibited dramatic differences. No two populations surveyed had identical genotypes or genotype frequencies. Two plains populations that were particularly interesting were CSP and FNWR because the CSP population contained two unique haplotypes and the Ft. Niobrara bison were fixed for one genotype. Populations of wood bison at EINP, MBS, and bison from WBNP also contained unique genotypes: 4, 5, 6a and 6b. Therefore, they too could be separated from other bison populations.

4.2.2 Wapiti

The eight restriction enzymes applied in this study provided 33 different fragments for the examination of intra- and inter-population variability. Three restriction fragment length polymorphisms were used to classify the haplotypes of 49 wapiti (Table 4.5). When the wapiti d-loop was digested with *Hinf I*, the 625 and 321 bp fragments from Roosevelt wapiti were separated from the 492, 321, and 133 bp fragments yielded by all other wapiti. This haplotype did not occur in any region sampled except the Olympic Peninsula and Vancouver Island. When the amplified DNA was digested with *Cfo I*, the fragments yielded were either 665, 385, and 286 bp or 450, 385, 286, and 230 bp in length. The first restriction pattern was expressed in all three subspecies while the second pattern, although infrequent, identified a third haplotype exclusive to wapiti in or near the Rocky Mountain Range.

4.3 Bison Populations with Unique Haplotypes

4.3.1) Custer State Park

While the variants at sites 429, 607, and 813 did not separate bison into wood and plains, they served to partition off haplotypes 1 and 2 from CSP. The variant at site 813, assayed with an allele-specific primer, separated haplotypes 2 and 3 from haplotypes 1, 4, 5, 6a, 6b, 7a, 7b, 8a, and 8b. A further separation of haplotypes 2 and 3 was achieved by restricting the d-loop mtDNA with *Sty I*. This enzyme recognized a variant at site 429 present in haplotype 3 and not in haplotype 2.

Although confirmation of haplotype 1 was pursued with several restriction enzymes, *Hinf I* provided the first clue to the unique haplotype. *Hinf I* identified the 607 site and yielded uncut or cut products. The uncut products separated haplotypes 1, 7a, 7b, 8a, and 8b from restricted products of haplotypes 4, 5, 6a, and 6b. The unrestricted products again were divided into two groups; haplotype 1 was separated from haplotypes 7a, 7b, 8a, and 8b using information from the *Sty I* digest. While similar in length, the haplotype 1 *Hinf I* fragments found in two CSP bison were slightly larger than *Hinf I* fragments of haplotypes 7a, 7b, 8a, and 8b (see Figure 4.0). These larger fragment lengths corresponded to restriction lengths given for cow. The larger fragments of the cow d-loop can be traced to insertions at sites 252 and 456, which are not present in the bison sequence. Bison expressing the cow-like haplotype (haplotype 1) shared restriction patterns with domestic cow for variants at sites 221, 289, 364, 365, 427, 446, 455, 655, and 813, but not at site 564. Both haplotypes 1 and 2 were confirmed by sequence data collected by Beech (unpublished).

4.3.2 Wood Buffalo National Park

While WBNP had the most diverse population of bison, expressing eight of the eleven haplotypes, the distribution of the haplotypes was not significantly different among sub-populations (contingency $X^2 = 14.350$ when not corrected for small sample size; $p>0.80$, d.f. 21, Spiess 1977). Pine Lake and Sweetgrass subpopulations contained the haplotypes 3, 4, 5, 6a, 6b, 7a,7b, and 8a of which genotypes 4, 6a, 7a, and 7b were missing from subpopulations at Garden Creek and Little Buffalo. The absence of haplotypes likely corresponds to the smaller sample size taken from Garden Creek and Little Buffalo. WBNP bison did not share haplotypes 7a or 8a with wood bison at EINP; haplotypes 4 and 6a with wood bison at MBS; and haplotype 7b with either wood populations. WBNP also did not share haplotypes 4, 5, 6a, and 6b with any of the plains bison populations. Because the haplotypes 3, 7a, 7b, and 8a found in WBNP were distributed across populations in North American, no plains haplotypes were unique to WBNP.

4.4 Cladistic Analysis of Bison Haplotypes

Using information from restriction analysis, only one parsimonious tree (Figure 4.1) was constructed for the eleven bison haplotypes using the heuristic searching method of PAUP 3.0. This tree was based on informative, unweighted characters and used domestic cow as an outgroup. The consistency index (CI) and branch length for the tree were 0.846 and 13 respectively. Reversals of nucleotides occurring along the branches for cow and group 6b at site 564; and along branches separating groups 1 and 2 at site 429, lowered the CI and increased the branch length. Because all other branch lengths had a CI of 1.00, bootstrapping was not performed to determine confidence levels.

From bison sequence data collected by Beech (unpublished), a maximum of fifteen transitions, two transversions, three insertions, and one deletion were identified among bison. Sites which are polymorphic and occur in at least two individuals are considered to be phylogenetically informative. There were 21 base pair changes in the bison d-loop with 4 uninformative sites (183, 364, 427 and 655) and 17 informative sites (221, 273, 278, 289, 365, 372, 374, 429, 446, 455, 472, 564, 607, 813, 846, 849, and 861). From primer-generated RFLP's, sites 364 and 655 were found to be informative as well. The branching pattern of Figure 4.2 (strict), Figure 4.3 (semi-strict), and Figure 4.4 (majority-rule) identify the consensus trees of 80 equally parsimonious trees (length = 29 steps, CI 0.759) analyzed from the sequence data by PAUP 3.0.

The phylogenetic tree, derived from the consensus of restriction data, was used to illustrate the lineages of bison. All bison were derived from an ancestor that gave rise to both wood and plains types. Bison were classified using Farris's (Wiley 1981) method of deriving paraphyletic and polyphyletic groups, where paraphyly describes members in which a reversal of a character has occured, and polyphyly describes members in which characters were not uniquely derived (parallel or convergent). To determine the classification of plains bison (Figure 4.5), all branches representing plains bison haplotypes were assigned a group membership number 1. Branches representing the ancestral or out-group and wood bison haplotypes were assigned values of 0. The plains bison group was considered a paraphyletic group because not all lineages were considered part of the group, and moreover, reversals occurred in two lineages.

The classification of wood bison (Figure 4.6) was also determined using this algorithmic method, except the wood bison groups were assigned the number 1 and then scored along branches to identify reversals or convergent evolution. The out-group and any branches representing plains bison haplotypes were assigned 0. From the tree, we see that wood types have evolved at least three non-unique characters in parallel with

plains bison, suggesting they constitute a polyphyletic group. If the wood bison group consisted of only lineages 4, 5, 6a, and 6b, they would have constituted a monophyletic group.

4.5 Genetic Distance Between Bison Populations

Estimates of the mean pairwise differences between individuals within each park were calculated using Equation 3.1 (Table 4.6). These values indicating intrapopulational distances, ranged from 0.000 for FNWR to 2.684 for CSP. The average variations within wood, plains, and WBNP bison populations were 2.129, 2.202, and 2.495, respectively. With the exclusion of FNWR, none of the distances among haplotypes within parks were significantly different.

Estimates of total pairwise differences and the maximum genetic distances between bison populations, were derived from Equations 3.2 and 3.3 (Tables 4.6 and 4.7, respectively). The phenetic tree derived from UPGMA (Figure 4.7) compared the inter-population distances of bison using equation 3.3, which corrects for intra-population variation. The most similar populations were NBR and CSP (d= 0.143); and WBNP and MBS (d= 0.146), while the least similar populations were FNWR and EINP woods (d= 3.388); and YNP and EINP woods (d= 2.506). Populations originating from WBNP are grouped closely together, while plains populations, all of which were derived from various mixtures of individuals from a small number of founding populations, show no such relationship. This variation found within plains bison is largely influenced by their intrapopulation variation and small number of founders. The populations with the least intrapopulation variation, such as FNWR and YNP bison, were the most distant from either wood or plains population, where the distance between these two groups equals: d= 1.536.

4.6 Divergence

Divergence between bison was calculated from restriction data using Equations 3.4 to 3.7. The bison were classified as having or not having the *Hinf I* site, therefore divergence was not calculated as a separation of woods and plains. Values of divergence between the haplotypes 2, 3, 4, 5, 6a, and 6b from 7a, 7b, 8a, and 8b ranged from 5,299 to 132,475 years depending on the rate of nucleotide substitution. From the haplotype information, the number of differences between wood and plains types can increase from one to six differences, therefore the divergence can be increased to 794,850 years.

The time of divergence, calculated between wapiti subspecies using Equations 3.8 to 3.11, was estimated between wapiti haplotypes 1 and 3, and between 1 and 2 to be approximately 3,423 to 160,000 years when $h = 2.0$ substitutions/bp/10^6yr and $h = 0.08$ substitutions/bp/10^6yr. The time of divergence between haplotypes 2 and 3 ranged from 4,065 to 406,500 years using the rates of substitution given above. This suggests the mutations were most likely accumulated before the wapiti became established in the different North American ecozones.

Figure 4.0 Restriction patterns of bison d-loop mtDNA using restriction enzymes *Ssp I*, *Sty I*, and *Hinf I* , whereby *Ssp I* recognizes sites 221, 289, and 472 with primer set 2/25 and site 364 with primer set 155/25. The restriction patterns of: *Ssp I* distinguished haplotypes 5 and 8b from other haplotypes (OH) at sites 221 and 289, *Ssp I* differentiated haplotype 6a from OH at site 472, *Sty I* separated haplotypes 1 and 2 from OH at site 429, and *Hinf I* separated haplotypes 7a, 7b, 8a, and 8b from 1 and OH (including 2) at site 607. Note in the restriction patterns of *Sty I* and *Hinf I*, the fragments of haplotype 1 are slightly larger than haplotypes 2.

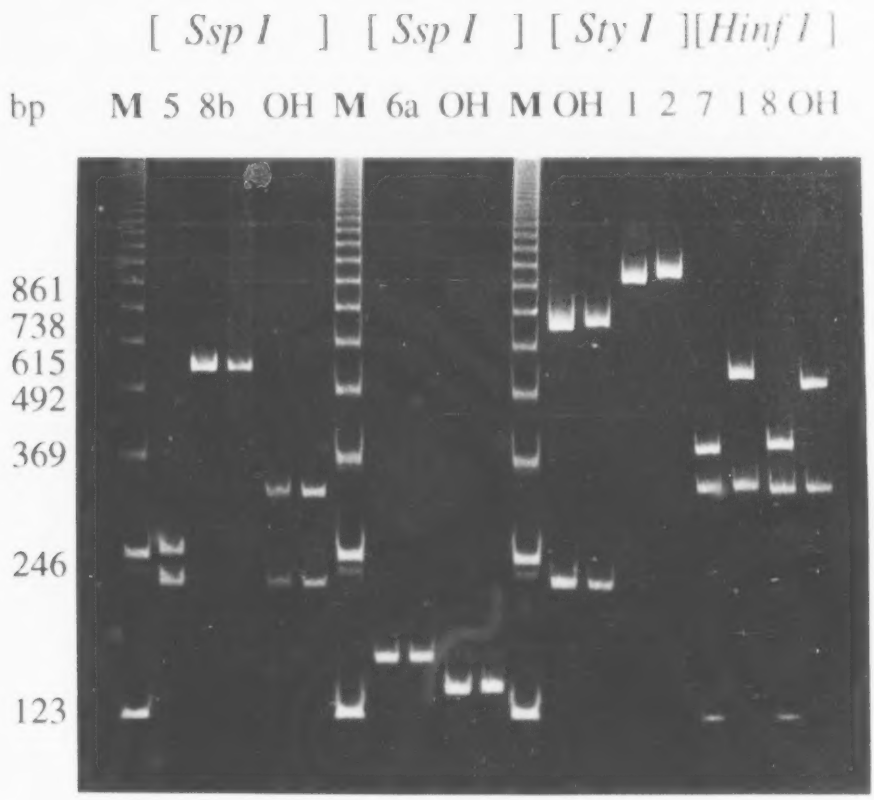

[Ssp I] [Ssp I] [Sty I][Hinf I]
bp
M 5 8b OH M 6a OH M OH 1 2 7 1 8 OH
861
738
615
492
369
246
123

Table 4.0 Haplotype frequencies (%) observed in bison populations from North America.

Park	Haplotype 1	2	3	4	5	6a	6b	7a	7b	8a	8b	n
Yellowstone N.P.	-	-	25.0	-	-	-	-	-	-	-	75.0	12
National B.R.	-	-	31.8	-	-	-	-	13.6	13.6	-	40.9	22
Custer S.P.	6.7	30.0	26.7	-	-	-	-	-	3.3	-	33.3	30
Ft. Niobrara	-	-	-	-	-	-	-	-	-	100.0	-	20
Wichita Mts.	-	-	5.0	-	-	-	-	55.0	-	40.0	-	20
Elk Island N.P												
(plains bison)	-	-	52.5	-	-	-	-	2.5	15.0	12.5	17.5	40
(woods bison)	-	-	22.2	8.9	26.7	20.0	22.2	-	-	-	-	45
Mackenzie B.S.	-	-	4.5	-	36.4	-	18.2	9.1	-	31.8	-	22
Wood Buffalo	-	-	17.2	6.9	22.4	8.6	17.2	3.4	10.3	13.8	-	58
(Pine Lake)	-	-	-	19.0	4.8	33.3	9.5	14.2	-	9.5	9.5	21
(Sweetgrass)	-	-	14.3	14.3	9.5	14.3	9.5	9.5	14.2	14.2	-	21
(Little Buffalo)	-	-	12.5	-	25.0	-	37.5	-	12.5	12.5	-	8
(Garden Creek)	-	-	25.0	-	25.0	-	25.0	-	-	25.0	-	8

Table 4.1 Observed number of individuals of each haplotype in bison populations from North America.

Park	Haplotype 1	2	3	4	5	6a	6b	7a	7b	8a	8b	total
Yellowstone N.P.	0	0	3	0	0	0	0	0	0	0	9	12
National B.R.	0	0	7	0	0	0	0	3	3	0	9	22
Custer S.P.	2	9	8	0	0	0	0	0	1	0	10	30
Ft. Niobrara	0	0	0	0	0	0	0	0	0	20	0	20
Wichita Mts.	0	0	1	0	0	0	0	11	0	8	0	20
Elk Island N.P.												
(plains bison)	0	0	21	0	0	0	0	1	6	5	7	40
(woods bison)	0	0	10	4	12	9	10	0	0	0	0	45
Mackenzie B.S.	0	0	1	0	8	0	4	2	0	7	0	22
Wood Buffalo N.P.	0	0	10	4	13	5	10	2	6	8	0	58
(Pine Lake)	0	0	4	1	7	2	3	0	2	2	0	21
(Sweetgrass)	0	0	3	3	2	3	2	2	3	3	0	21
(Little Buffalo)	0	0	1	0	2	0	3	0	1	1	0	8
(Garden Creek)	0	0	2	0	2	0	2	0	0	2	0	8
total	2	9	61	8	33	14	24	19	16	48	35	269

Table 4.2 Haplotype frequencies (%) observed in wapiti populations from North America.

Population	Location	n	Haplotype 1	2	3
Roosevelt	Olympic	8	-	-	100
	Vancouver	8	-	-	100
Rocky Mountain	Banff	11	72.7	27.3	-
	Jasper	4	100	-	-
	Rocky Mountains	3	100	-	-
Manitoban	Elk Island	11	100	-	-
	Duck Mountain	4	100	-	-

Table 4.3 Observed number of individuals of each haplotype in wapiti populations from North America.

Population	Location	Haplotype 1	2	3	total
Roosevelt	Olympic	-	-	8	8
	Vancouver	-	-	8	8
Rocky Mountain	Banff	8	3	-	11
	Jasper	4	-	-	4
	Rocky Mountains	3	-	-	3
Manitoban	Elk Island	11	-	-	11
	Duck Mountain	4	-	-	4

Table 4.4 Restriction fragment length polymorphisms found in the bison d-loop.

Enzyme	Sequence Recognized	Variant	Fragment Lengths (bp)
Alu I	AG ' CT	A	497, 245, 144, 94, 92
Cfo I	GC ' GC	A	1113
Dde I	C ' T(A/T)AG	A	1113
Hae III	GG ' CC	A	531, 248, 155, 116, 63
Hinf I	G ' ANTC	A	607, 506
		B	1113
Mae I/Bfa I	C ' TAG	A	317, 268, 233, 192, 103
		B	420, 268, 233, 192
Rsa I	GT ' AC	A	710, 206, 115
Sty I	C' CTAGG	A	684, 429
		B	1113
Taq I	T ' CGA	A	641, 472

Table 4.5 Restriction fragment length polymorphisms found in the wapiti d-loop.

Enzyme	Sequence Recognized	Variant	Fragment Lengths (bp)
Alu I	AG ' CT	A	342, 275, 205, 110, 96, 91, 86
Cfo I	GC ' GC	A	665, 385, 286
		B	450, 385, 286, 230
Dde I	C ' T(A/T)AG	A	765, 282, 127, 76
Hae III	GG ' CC	A	620, 455, 150
Hinf I	G ' ANTC	A	492, 321, 133
		B	625, 321
Hpa II	C ' CGG	A	620, 455, 235
Rsa I	GT ' AC	A	765, 270
Sau 3A	' GATC	A	560, 369, 315
Taq I	T ' CGA	A	735, 518

Table 4.6 Total intra and inter pair-wise comparisons between haplotypes from North American bison populations.

	YNP	NBR	CSP	WMWR	FNWR	MBS	WBNP	EINPP	EINPW
YNP	**1.875**								
NBR	2.591	**2.554**							
CSP	3.217	2.762	**2.684**						
WMWR	2.400	2.182	2.787	**1.150**					
FNWR	1.750	2.364	3.267	1.300	**0.000**				
MBS	3.477	3.062	3.388	2.327	2.727	**2.496**			
WBNP	3.905	3.226	3.154	2.607	3.466	2.651	**2.514**		
EINPP	3.044	2.632	2.828	2.265	2.775	2.925	2.834	**2.380**	
EINPW	4.389	3.330	3.481	3.089	4.333	2.703	2.377	3.025	**1.890**

Table 4.7 Minimum genetic distance between haplotypes from North American bison populations using pair-wise comparisons.

	YNP	NBR	CSP	WMWR	FNWR	MBS	WBNP	EINPP
NBR	0.376							
CSP	0.937	0.143						
WMWR	0.887	0.512	0.870					
FNWR	0.812	1.087	1.925	0.725				
MBS	1.282	0.528	0.789	0.495	1.470			
WBNP	1.720	0.701	0.564	0.784	2.218	0.146		
EINPP	0.916	0.165	0.296	0.500	1.585	0.478	0.396	
EINPW	2.506	1.108	1.194	1.569	3.388	0.501	0.184	0.890

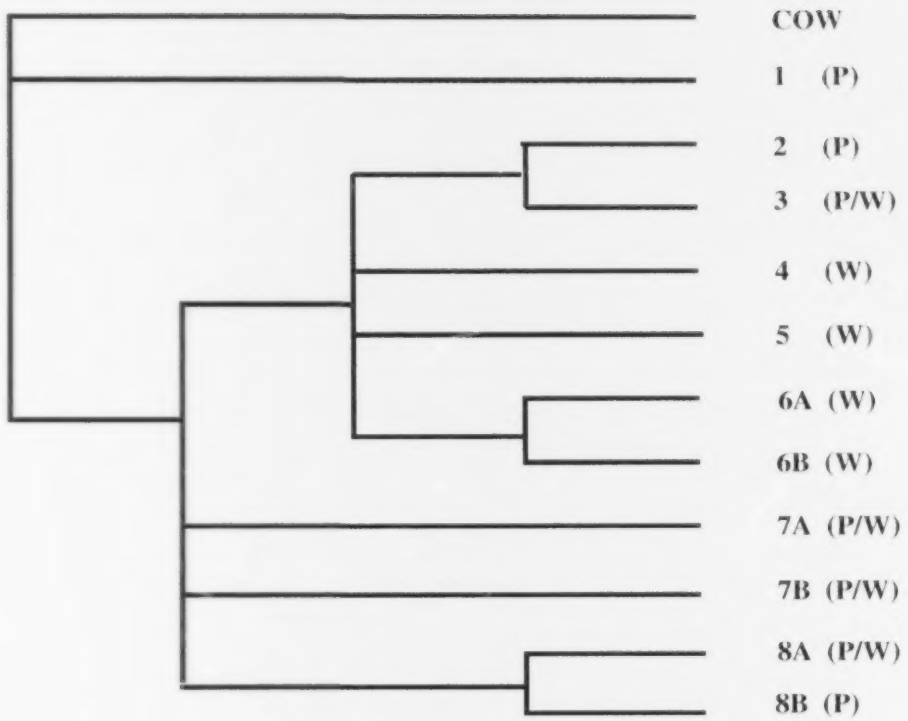


Figure 4.1 A phylogram resulting from parsimony analysis of mtDNA d-loop haplotypes that depicts the relationships within bison. Haplotypes 1 through 8b were identified by restriction analysis at 12 sites and correspond to haplotypes described elsewhere. Haplotypes found in individuals recognized as wood or plains bison were designated as wood (W) and plains (P). The tree has a branch length of 13 and CI of 0.846. Domestic cow was identified as the outgroup.

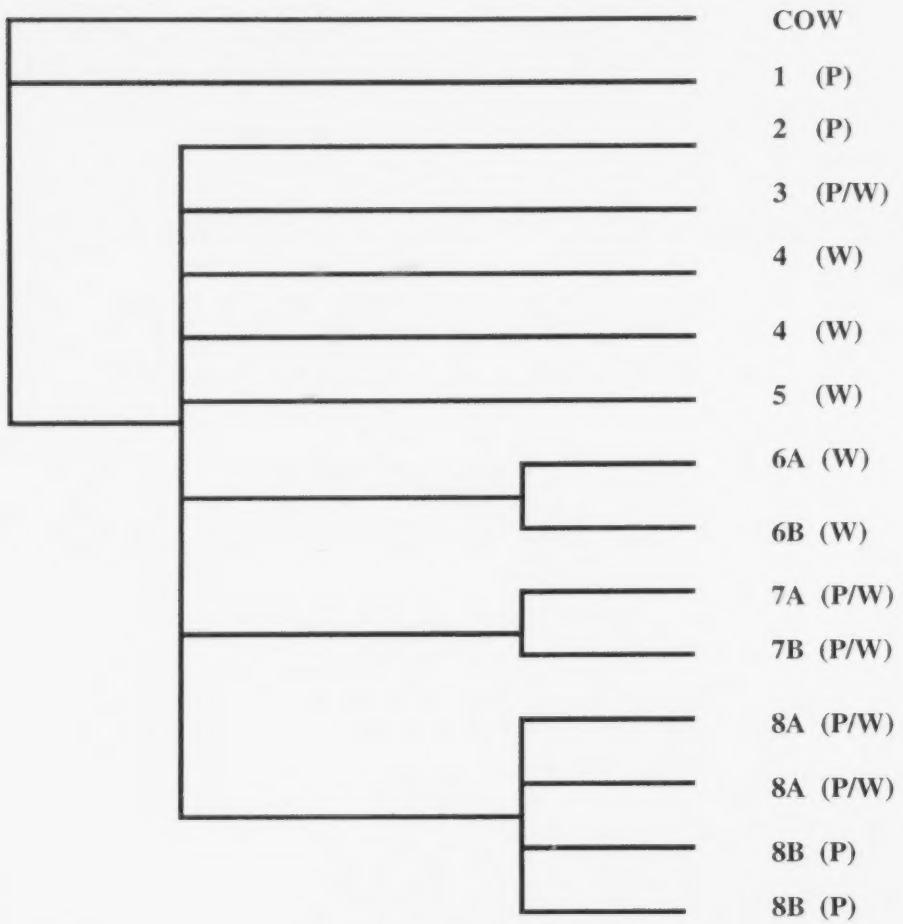


Figure 4.2 A phylogram depicting the relationships within bison haplotypes resulting from a strict consensus of 80 most parsimonious trees. These trees were determined by sequence data of 23 bison separated by 16 characters. The sequenced individuals were then assigned to haplotype groups identified using restriction data, thereby producing a phylogram with more than one branch for each haplotype group. The tree has CI = 0. 243, and uses domestic cow as an outgroup. Haplotypes shared by wood (W) and plains (P) bison are illustrated.

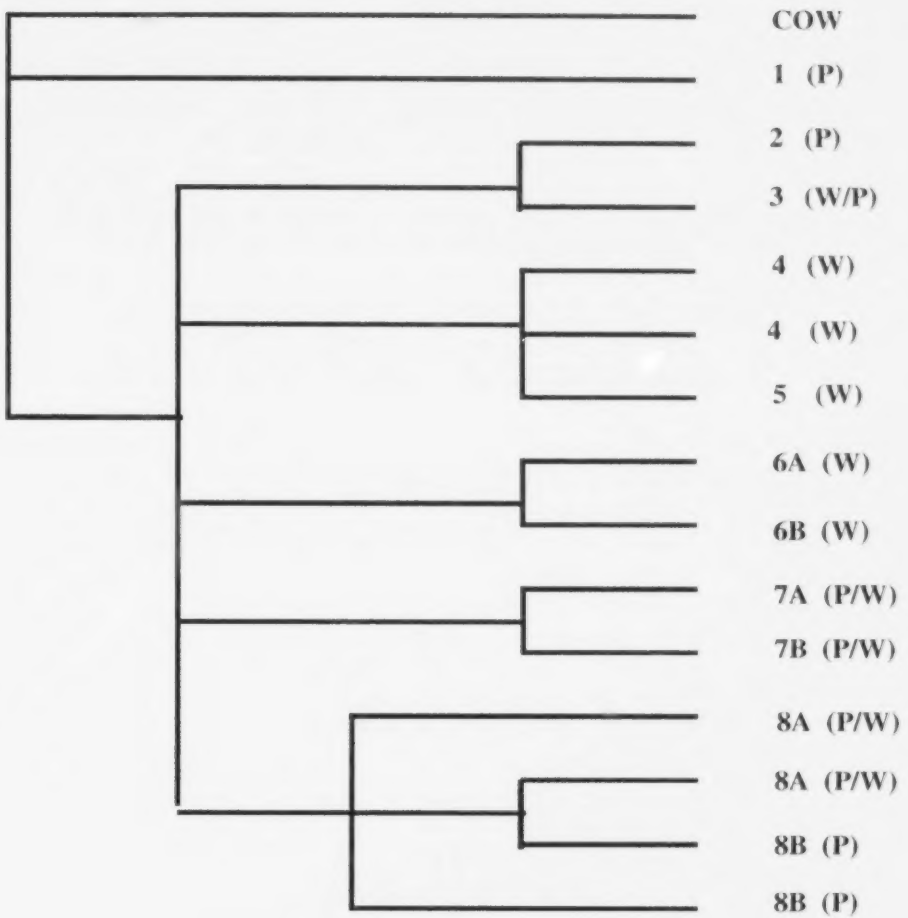


Figure 4.3 A phylogram resulting from a semi-strict consensus of 80 most parsimonious trees depicting the relationships within bison haplotypes. These trees were determined by sequence data of 23 bison separated by 16 characters. The sequenced individuals were then assigned to haplotype groups identified using restriction data, thereby producing a phylogram with more than one branch for each haplotype group. The tree has CI = 0. 553, and uses domestic cow as an outgroup. Haplotypes shared by wood (W) and plains (P) bison are illustrated.

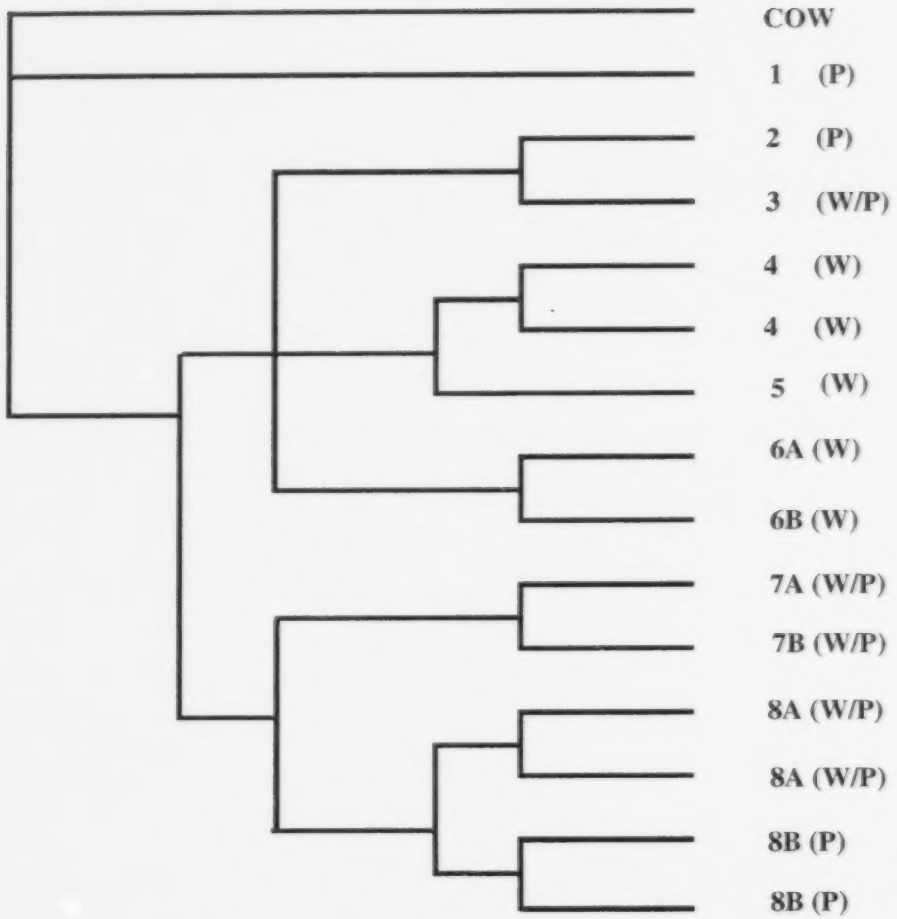


Figure 4.4 A phylogram resulting from a 50% majority rule consensus of 80 most parsimonious trees depicting the relationships within bison haplotypes. These trees were determined by sequence data of 23 bison separated by 16 characters. The sequenced individuals were then assigned to haplotype groups identified using restriction data, thereby producing a phylogram with more than one branch for each haplotype group. The tree has CI = 0. 895, and uses domestic cow as an outgroup. Haplotypes shared by wood (W) and plains (P) bison are illustrated.

Figure 4.5 A systematic classification of plains bison. Based on Farris's (Wiley 1981) algorithmic method of describing a phylogeny, the step-by-step assigning of group character memberships is indicated for the phylogram obtained from restriction data. Wood and plains bison are indicated by W and P, respectively. Character membership group numbers for each branch were assigned as: 1 for plains bison, 0 for non-plains bison, and B if both groups of bison were represented. Characters B were reassigned with a 0 or 1, depending on the character assigned to the most recent common ancestor. The reversal of one or more characters from 0 -> 1 -> 0 indicate that not members of the recent ancestor are included in the plains bison group, indicating plains bison are paraphyletic.

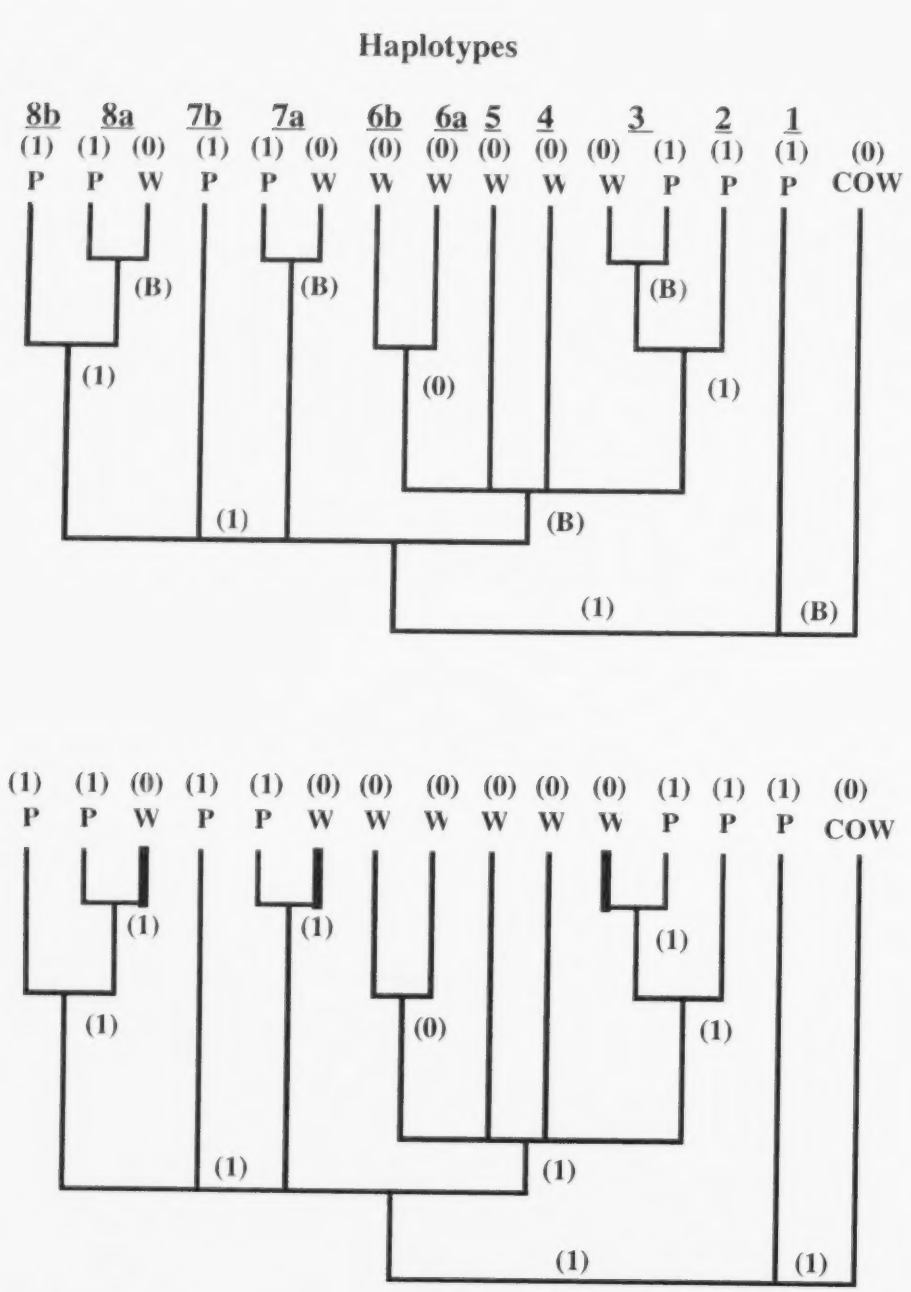
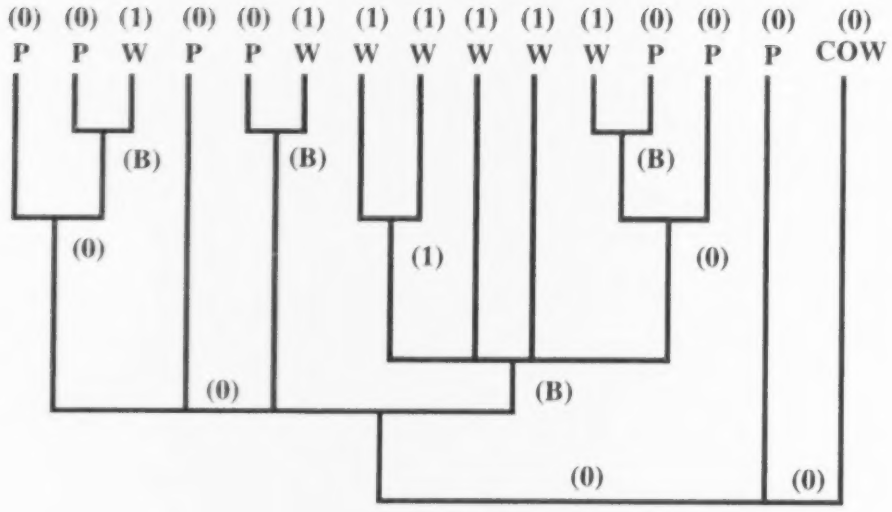
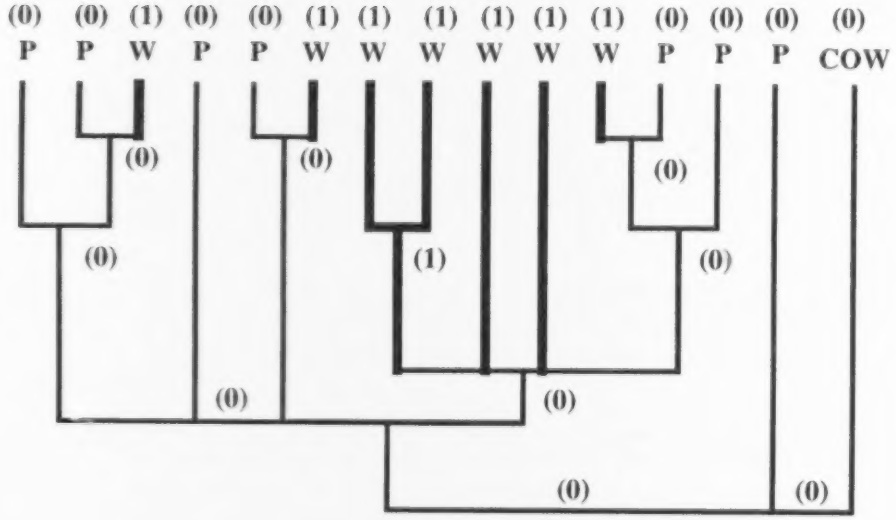

Haplotypes
8b 8a 7b 7a 6b 6a 5 4 3 2 1
(1) (1) (0) (1) (1) (0) (0) (0) (0) (0) (0) (1) (1) (1) (0)
P P W P P W W W W W W P P P COW
(B)
(B)
(B)
(1)
(0)
(1)
(1)
(B)
(1)
(B)
(1) (1) (0) (1) (1) (0) (0) (0) (0) (0) (0) (1) (1) (1) (0)
P P W P P W W W W W W P P P COW
(1)
(1)
(1)
(1)
(0)
(1)
(1)
(1)
(1)
(1)

Figure 4.6 A systematic classification of wood bison. Based on Farris's (Wiley 1981) algorithmic method of describing a phylogeny, the step-by-step assigning of group character memberships is indicated. Wood and plains bison are indicated by W and P, respectively. Character membership group numbers for each branch were assigned as: 0 for plains bison, 1 for non-plains bison, and B if both groups of bison were represented. Characters B were reassigned with a 0 or 1, depending on the character assigned to the most recent common ancestor. The presence of one or more characters which are not uniquely derived indicate wood bison are derived from a polyphyletic lineage.


(0) (0) (1) (0) (0) (1) (1) (1) (1) (1) (1) (0) (0) (0) (0)
P P W P P W W W W W W P P P COW
(B)
(B)
(B)
(0)
(1)
(0)
(0)
(B)
(0)
(0)


(0) (0) (1) (0) (0) (1) (1) (1) (1) (1) (1) (0) (0) (0) (0)
P P W P P W W W W W W P P P COW
(0)
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(0)
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(1)
(0)
(0)
(0)
(0)
(0)

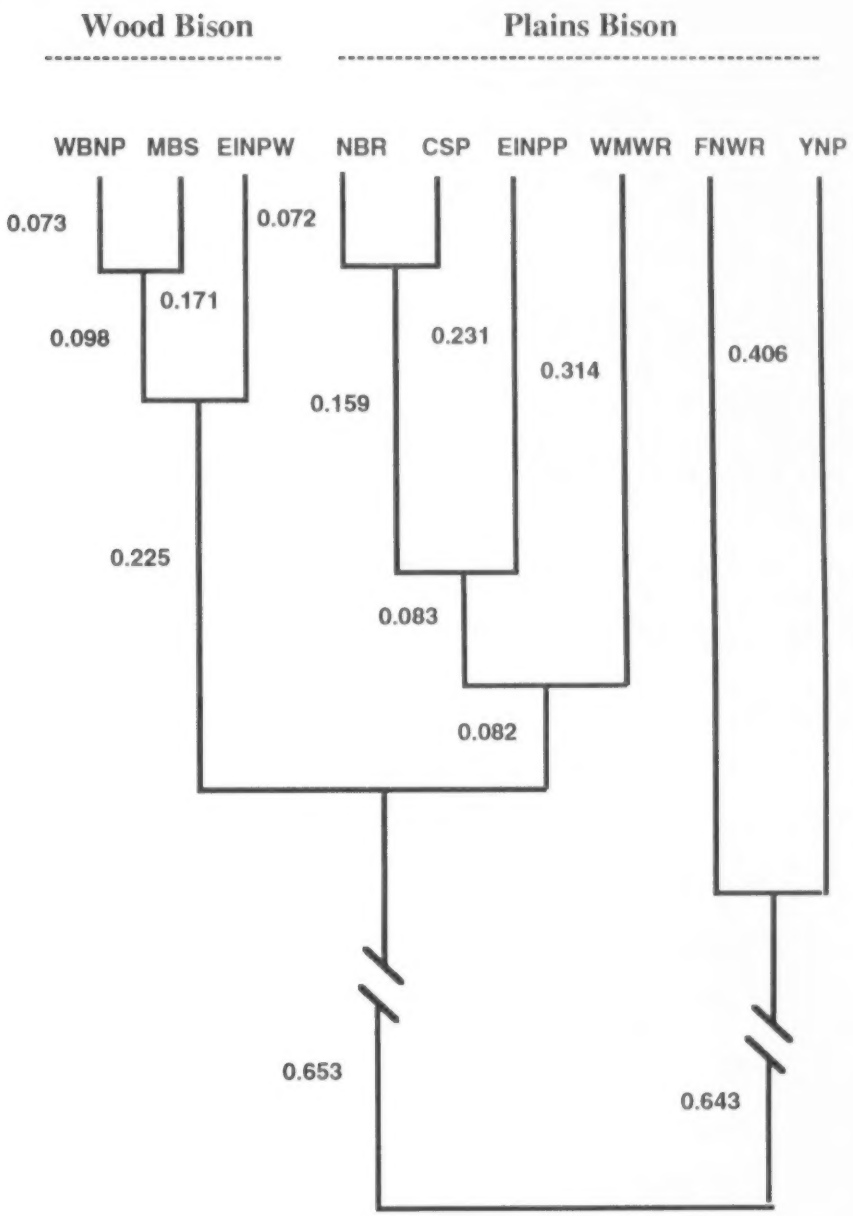

Figure 4.7 Genetic distances between North American bison populations based on UPGMA analysis of haplotypes identified by restriction analysis of 12 characters.

Chapter 5
Discussion

The application of molecular technology to answer population and taxonomic questions is beginning to be realized. The technology applied will depend on the information needed. The more similar the genotypes of the taxa, the more variable the region of DNA must be to distinguish between them. Identification of differences between taxa, such as between species, can be performed by simply restricting PCR products with endonucleases and observing restriction fragment length polymorphisms (RFLPs). Studies to observe genetic variation within and among populations, however, usually require a combination of sequencing data and PCR technology. Information from sequencing allows lineages for conspecifics to be identified, variable sites to be located, and primers to be designed to survey these sites. While the survey does not provide as detailed information as the sequence data, the primer/restriction enzyme combination provides information regarding the composition and relationships between populations. Populations that are not only small but extremely similar, such as polar bears (*Ursus maritimus*), can be differentiated using DNA fingerprinting. DNA fingerprinting involves the identification of genotypes based on the presence of highly repetitive nucleotide sequences. These abundant repetitive sequences, also known as variable tandem repeats, mini-satellites, or micro-satellites, are randomly dispersed in the genome and inherited in a Mendelian fashion.

5.0 Implications of genetic variability drawn from the mt DNA d-loop

The first studies to look for genetic variation using protein electrophoresis concluded that large mammals had an impoverished gene pool (Ryman *et al.* 1970;

Bonnel and Selander 1974; Sage and Wolff 1983). Several theories were developed to explain the lack of diversity. Protein electrophoretic data from natural populations suggested that large, highly mobile animals, such as black bears (*U. americanus*), wapiti, seals (*Phoca*), moose and polar bears, exhibit lower levels of heterozygosity than small, less mobile animals because they can maintain a higher biological homeostasis. This implies large mammals can move, within a reasonable time, to areas with more shelter or forage (Selander and Kaufman 1973; Ryman *et al.* 1980). Greater genetic diversity in small, less mobile mammals would seem logical if they were to survive changes in their environment (Wooten and Smith 1985). Baccus *et al.* (1983) and Gyllensten *et al.* (1983) however found no correlation between body size and heterozygosity. In fact, white-tailed deer, which are habitat generalists, have a high degree of heterozygosity (Baccus *et al.* 1983; Kennedy *et al.* 1987).

Differences in the genetic diversity of large mammals may also be influenced by social structure. For example, Gyllensten *et al.* (1980) found populations of red deer, which form harems and live in overlapping territories, have a greater number of transferrin alleles than moose or roe deer (*Capreolus capreolus*), which lead a more solitary existence. Transferrin is a protein that helps fight infectious diseases which are more likely to occur in dense populations (Gyllensten *et al.* 1980).

Perhaps the lack of variation was not due to an absence of variability in the populations, but to the inability to locate and analyze the differences. Population studies have been given a new future with the advancement of molecular technology. Early protein electrophoretic studies, which provided us with the first clues to the diversity among populations, underestimated the variation in mammals (Bargiello *et al.* 1977; Quinn and White 1987). Molecular techniques available today include the polymerase chain reaction (PCR), sequencing, and DNA fingerprinting.

Variation in the d-loop region of mtDNA, as determinined by RFLPs, sequence data, or both, can be used to discriminate between taxa and among lineages within taxa. For example, wapiti, bison, and domestic cattle are separated by specific nucleotide differences, and each taxa is further subdivided into its respective lineages by unique nucleotide changes. In this study, each lineage or haplotype was represented by a specific set of restriction patterns which reflected changes in the nucleotide sequence. Most nucleotide variations in the bison haplotypes were traced to transitional changes, or changes between purines (guanine; G and adenine; A) or pyrimidines (thymine; T and cytosine; C). Among closely related species, the majority of changes will be transitions, while among more distant taxa, the number of transversions or nucleotide changes between A and T, or G and C will increase (Brown *et al.* 1979; Kocher *et al.* 1989). Based on sequence data (Beech unpublished), the number of transitions and transversions observed between bison and domestic cow were 59 and 24, respectively, and between bison and bison were 15 and two, respectively. In addition, three insertions and one deletion place bison and cow significantly apart from each other. Specific nucleotide changes could not be directly determined from restriction site analysis in this study, although, from primer-generated restriction analysis, up to eleven sites could be surveyed for variation which identified a maximum of six nucleotide changes between bison haplotypes (Table 3.4).

Although less than 13 % of the nucleotides in the wapiti d-loop were sampled for variation using restriction enzymes (calculated from the sum of nucleotides recognized by the restriction enzymes), the actual number of nucleotide changes between haplotypes must be greater. The use of primer-generated restriction sites in the bison d-loop analysis demonstrated that restriction enzymes in conjunction with specially designed primers will identify additional polymorphic sites and genotypes (Tables 4.1). Therefore, more than three haplotypes must exist in wapiti populations.

Wildlife conservationists are concerned with the amount of genetic variability in a population, subspecies, or species. A previous study by Cronin (1986) revealed little to no variation in total restricted mtDNA's from bison and wapiti. In the present study, however, a number of haplotypes were identified in these populations using PCR technology. Although one can argue that more sensitive assays should reveal more variation, the additional information gained by these techniques can also provide greater insight into a population.

The diversity of the gene pool of a population will influence its survival. Monoculture crops, which have a high susceptibility to disease and low adaptability to environmental changes, demonstrate two significant problems that result from a lack of genetic diversity. A similar absence of genetic diversity was observed in cheetah populations (O'Brien 1989). Although the population size has recovered and the cheetah expresses normal fertility and age expectancy (Lindburg and Worley 1993), the long term survival of the cheetah (*Felis concolor coryi*), which is dependant on the genetic diversity, is still questionable. Because the integrated plains and wood bison at WBNP represent the most genetically diverse population of bison, opposition to the slaughter of these bison can be understood.

O'Brien and Mayr (1991) pointed out that the survival of a species depends on maintaining a diverse gene pool. Efforts to conserve a species, therefore, should focus on its populations. Steps can be taken to increase a population's gene pool or to maintain the 'status quo' through the introduction of genotypes from another population or by conserving an adequate number of individuals in the population. Although numbers alone are not enough to preserve the genetic diversity of a population, larger populations are less affected by changes in gene frequencies, and require greater periods of time for genotypes to be lost.

As information is gathered regarding the genetic diversity of populations, it should be possible to re-introduce individuals to areas without significantly altering existing populations. If the lineages of populations are known, the effect an introduction of individuals would have on the existing gene pool could be determined before a relocation was performed. If the populations share similar genotypes, then the effect will be minimal. For example, the introduction of bison from Ft. Niobrara Wildlife Refuge into Yellowstone National Park would have less impact on the gene pool than an introduction of bison from the Mackenzie Bison Sanctuary. Likewise, moving wapiti from the prairies to Pacific coast would significantly affect the gene pool of Roosevelt wapiti.

The question can then be raised, "Should we be altering gene pools of populations, and if we do, will the result be positive or negative?" Although the introduction of genotypes will have less effect on the gene pool of large populations than on small populations, the integration of genotypes may lead to a population with a greater ability to absorb changes in its environment. The introduction of individuals from a population that is going extinct into larger related populations, allows us to salvage some of its genetic information. However, the introduction of new genotypes into a native population will destroy the ability to determine the bio-geographic history of a population.

The management of populations for the reason of maintaining subspecies, however, may be futile when the direction that any subspecies may take is to: (i) go extinct; (ii) exchange genes with another subspecies and become a new mixed subspecies; (iii) change its genetic character over time to become one or more new subspecies by genetic drift, selection, subdivision, or other demographic processes; and (iv) become a new species if effectively isolated (O'Brien and Mayr 1991).

5.1 Intra-population variability

As a result of the population bottlenecks historically experienced by North American bison and wapiti, present populations probably do not reflect the genetic diversity that once existed. The effects of genetic drift, such as the loss or fixation of an allele in a randomly mating population will be greater for mtDNA than for nuclear DNA, and are more pronounced in populations with few founders. Therefore, the haplotype frequencies should be different between herds of bison or wapiti, and prove useful for distinguishing populations from one another.

Different haplotype frequencies suggest that either different selection pressures were acting in each population, different founding members had contributed to each founding stock, or an error had been made in sampling. The haplotypes of bison populations ranged from being fixed (FNWR) to varied, even in populations with similar founders as in wood bison from EINP and MBS.

The difference in haplotype frequencies was compatible with the history of the founding populations. Both EINP and MBS wood bison originated from an isolated herd at WBNP, yet these three populations all expressed slightly different frequencies Table 4.0). The difference in haplotype frequencies at EINP and MBS likely arose from selecting founding members for each park over two separate sampling periods, whereby the founding members did not have equal haplotype frequencies.

Bison used to establish herds varied in origin and number; ranging from eight bison donated from one ranch to FNWR to more than 50 bison of several origins founding YNP. Only WBNP began with a reasonably sized population of approximated 250 indigenous animals (Federal Review 1989). Therefore, one may expect bison at WBNP to have the greatest diversity in haplotypes and FNWR to have none (Table 4.0). Likewise, populations such as the Roosevelt wapiti, isolated in one

geographic area should have genotypes that set them apart from populations whose range is less defined. The greater diversity of Rocky Mountain wapiti may reflect a combination of populations indigenous to the area, introductions of wapiti from YNP, RMNP and EINP, and a hybridization with Manitoban wapiti.

Sampling error may result when samples are taken from individuals in only one sub-population or population in only one area of a range, because the samples may not represent the entire population if extensive gene flow is not occurring between herds. Bork (1990) inferred from negative inbreeding coefficients that although the bison populations at EINP have experienced genetic bottlenecks in the past, the existing genetic information within each population was evenly distributed by random mating. The similar distribution of d-loop haplotypes in the four sub-populations in WBNP (Table 4.0) also supports equal distribution of genetic information as a result of random mating.

As defined by restriction patterns of the d-loop mtDNA, the average number of haplotypes found in northern and plains bison populations was 6 and 3.3, respectively (Table 4.1). A distinction was made between wood and northern bison, whereby northern bison included populations with haplotypes 4, 5, 6a, and 6b found only at WBNP, MBS and EINP. The term wood bison was used when acknowledging bison populations formally recognized as wood bison, and existing only at MBS and EINP. The exclusion of WBNP bison from the wood bison group is incorrect because these bison generally express the same haplotypes as bison in MBS or EINP, although at different frequencies.

The wapiti populations exhibited little-to-no variation within their populations. Similar to bison, wapiti herds also share the problem of having few founding members, with the exception of YNP, which was estimated to have supported several thousand wapiti even at the turn of the century. The wide distribution of a single genotype may

also reflect genetic bottlenecks and extensive gene flow between founders; however, a more plausible explanation is that much genetic variation was undetected using restriction enzymes.

The Manitoban wapiti populations sampled from EINP and DMPP fall into the overlapping region of the Rocky Mountain wapiti. Therefore, all wapiti populations sampled from the Rocky Mountains and eastward may actually represent one subspecies. Because the EINP and DMPP wapiti populations were reminiscent of the original eastern herds, the possibility exists that only one subspecies of wapiti predominates in the Rocky Mountains and eastward.

Analysis of the haplotypes found within bison and wapiti populations confirms that populations can be distinguished from each other because they are evolving as unique entities. In addition, haplotypes tell us something about the history, distribution and evolution of populations. Northern bison populations were found to express a set of haplotypes that suggest that at one time, wood bison existed as an isolated population. The introduction of plains bison to WBNP, unfortunately, has made the task of identifying the original WBNP genotypes impossible. The presence of a unique haplotype in CSP indicates genetic variation was common to bison populations across North America, while the universal haplotype suggests that gene flow was not restricted between bison populations. Finally, observations of the haplotypes in CSP also revealed the unfortunate cross-breeding event between cattle and bison which likely occurred long before their introduction to the park.

5.2 Inter-population variability

After the rapid decline in their numbers, the belief that bison were largely inbred was supported by low values of heterozygosity, where average H ranged between

0.023 and 0.012 (Wurster and Benischke 1968; McCleneghan *et al.* 1990). Although heterozygosity depends on the size and location of a population (Ryman *et al.* 1977; Baccus *et al.* 1983), values given for bison were equivalent to values determined for other large indigenous and circumpolar mammals. Average heterozygosity ranged from 0.014 to 0.017 for moose, North American wapiti, and caribou, whereas values for red deer, mule deer (*Odocoileus hemionus*), and white-tailed deer (*O. virginianus*) ranged as high as 0.047 to 0.074 (Baccus *et al.* 1983).

Most bison populations have few polymorphic loci, and loci that are polymorphic in one population may be invariable in another. For example, bison at WMWR were polymorphic for locus 6-PGD, a monomorphic site for bison from Badlands National Park, South Dakota which is a population that is polymorphic for only one of 24 loci (McCleneghan *et al.* 1990). The wood and plains bison at EINP were polymorphic at only 2 of 50 restriction site loci of four growth regulating genes (Bork *et al.* 1990). The lack of variation at these loci likely represents the conservative nature of growth-regulating genes and large number of monomorphic loci. The large number of monomorphic loci is not limited to bison, as Ryman *et al.* (1980) found only 5 of 23 loci to be polymorphic for moose in Scandinavia.

The variation found within the d-loop region identified several genotypes within a population. Recognizing as many genotypes as possible is important when trying to establish the extent to which similar populations have diverged from each other. To infer the relationship between populations, the genetic identity or genetic distance can be calculated between genotypes. Bork *et al.* (1991) found the genetic identity between wood and plains populations emphasized their relatedness (0.985), but was different enough to suggest they were unique populations. The genetic identity between the bison groups corresponded to that found in geographically isolated small mammal populations (0.933 - 0.983 Ayala 1975).

The genetic distances determined in this study were between nine bison populations expressing eleven haplotypes. These ranged from being common to all herds (haplotype 3) to being exclusive to individual parks (haplotypes 1 and 2). Haplotypes 4, 5, 6a, and 6b were unique to bison originating from WBNP. With the exception of haplotype 8b, the remaining haplotypes (7a, 7b, and 8a) were common, but not exclusive, to plains bison.

Genetic distance measurements indicated wood bison from WBNP and MBS were most related to each other (d = 0.146) and to bison at EINP (d=0.343). This is not surprising, since both populations originated from the same isolated population at WBNP. Plains bison that originated from the same founding populations, however, do not show this trend. Bison at CSP and the NBR were the most similar to each other (d = 0.143) and to EINP plains bison (d= 0.230); bison at WMWR completed the group at d = 0.525. CSP and NBR had the most haplotypes in the plains populations and likewise were grouped together, although FNWR was comprised of founders from NBR and not included in the main plains bison cluster.

Calculating the genetic distance from invariable populations, such as the FNWR and YNP, places these populations together and more distant from all other populations. Small sample size, sampling error, and intra-population variability can all influence the distance determined between populations. The distance between all bison populations, a result of few founding members, is small and insignificant.

Genetic variability between wapiti populations was also studied. Johnston (1968) examined the Eurasian red deer and the North American wapiti using blood serum protein electrophoresis, and found that he could not differentiate between the two groups. Dratch and Gyllensten (1983), however, found red deer, wapiti and F1-hybrids could be easily identified by high resolution isoelectric focusing of hemoglobin samples. Their studies also concluded that variation among red deer subspecies was

low and that individual populations had only a small fraction of the total gene diversity. Hartl and colleagues (1986) tested for 12 blood proteins in a related species and found only one polymorphism in a herd of fallow deer (*Dama dama*). Cronin (1986, 1992) failed to locate mtDNA characters that could discriminate between subspecies of red deer and wapiti. The three genotypes identified by Cronin (1992) did not separate Roosevelt wapiti from other North American wapiti subspecies. In this study, which focused on the d-loop region of mtDNA, Roosevelt wapiti haplotypes were distinguished from Rocky Mountain and Manitoban haplotypes (Tables 4.2 and 4.3).

Comparing populations provides an insight into which populations are most closely related. This can be useful when tracing the historical movement of animals. If haplotypes are unique to one area, they likely have had little to no contact with outside populations. The presence of genotypes within two populations would imply that movement had or is occurring between the two groups. Historical variation and distributions of populations can also be reaffirmed by analyzing DNA from museum samples (Kocher *et al.* 1989; Thomas *et al.* 1989).

5.3 Sub-specific variation

The application of molecular techniques to systematics has allowed the inference of species or subspecies trees from gene trees. In the absence of gene flow, taxa should be partitioning into groups by the time-dependent accumulation of nucleotide differences. Groups that share nucleotide differences can be classified as descendants from one lineage, and constitute a well-defined group. Because hybrid zones exist between subspecies, paraphyletic populations are accepted as subspecies as long as the majority of individuals share the most recent common ancestor (O'Brien and Mayr 1990). Implementation of genetic methodology to classify animals will come with

some resistance. Part of this problem lies in the lack of a working definition of a species. At present no description recognizes or includes all species types. Perhaps, it is in this weakness that molecular systematics and molecular evolutionary biology has the most to offer.

In the phylogenetic trees created from mtDNA d-loop haplotypes, wood and plains bison were not separated into distinct phylogenetic groups. Although four unique haplotypes or lineages exist in bison at WBNP, EINP, and MBS, the percent of wood bison expressing plains bison haplotypes were 44.8, 22.2, and 36.4, respectively. The high percent of these haplotypes in WBNP suggests that the plains bison became highly dispersed among the park population. The lower frequency of plains haplotypes in EINP and MBS suggests that the subpopulation at Nyarling River was relatively isolated (Soper 1941); however, a few plains bison had already established themselves in the area before founding wood bison were sampled. The integration of plains bison into the Nyarling River population was reconfirmed by the presence of tuberculosis in wood bison transferred from this region to EINP (Review Panel 1990). The presence of unique plains haplotypes in WBNP would imply plains haplotypes were indigenous to the park; however, their absence does not suggest the opposite. Unfortunately, the introduction of plains bison to WBNP has destroyed the ability to determine if the plains haplotypes were already present in the northern populations or if they were introduced.

As the populations presently exist, bison in WBNP, MBS and EINP share a common gene pool. The proposal to replace WBNP with 'pure wood' bison from EINP or MBS was based on the belief that populations at MBS and EINP consisted of a gene pool restricted to wood bison and the populations at WBNP comprised a gene pool integrated from both wood and plains animals. Given the results of the present study, this proposal now seems inappropriate.

The presence of northern genotypes in these parks, however, suggests that consideration should be given to manage them separately. Under the 1979 guidelines of the Committee on the Status of Endangered Wildlife in Canada (COSEWIC), geographically distinct populations require separate management (Bork 1989). Recognizing the populations which contain the northern haplotypes as "unique" implies they are geographically distinct, which raises some serious questions, especially when each individual is considered unique. "Uniqueness" should not be viewed in terms of a percentage of variability, but rather as a distribution of the haplotypes. The large proportion of northern haplotypes in only the MBS, EINP (wood), and WBNP populations suggests that some level of isolation has been achieved, while the additional presence of plains haplotypes establishes the fact that *Bison*, today, are not comprised of well defined subspecies.

The significance given to an individual's or a population's genetic variability depends on the size and health of the population. Individual variation has a greater importance in a small or unhealthy population. With this in mind, consideration should be given to harvesting or maintaining the gene pool of WBNP because the population is rapidly declining, and the 18 and 23 "wood" bison taken from WBNP, to establish populations at the MBS and EINP, represent such a small proportion of the gene pool in WBNP. The diverse gene pool of bison in WBNP cannot be replaced with bison from other parks.

The subspecific division of wapiti is closely correlated with the data generated from the genetic analysis presented here. Unique and fixed haplotypes in Roosevelt wapiti supports its subspecies status. The mixture of haplotypes in Manitoban and Rocky Mountain wapiti demands that further studies be performed to determine their status. The presence of two haplotypes in the two subspecies suggests that some

degree of genetic separation has been achieved, although the extent of differentiation cannot be determined.

Until a solid database is established for the rates of nucleotide variability in the different regions of DNA, the time since populations have diverged from each can only be roughly estimated. Divergence between the wood and plains bison was estimated between 5,229 - 132,475 years ago. The variability observed between wapiti haplotypes was used to estimate the time of divergence from each another. The divergence of wapiti haplotypes placed the separation at 3,422 - 160,000. These times compare with times of divergence of populations of North American moose, which Reuterwal and Ryman (1979) calculated to have diverged into groups between 28,000 and 11,000 Y.B.P.

5.4 Future considerations

The advance of molecular technology and application to wildlife or conservation biology, has compounded the questions that can be asked and answered, or the answers that can be confirmed. By restricting oneself to inquiries of bison and wapiti, several challenges remain, including: the verification of the bison phylogeny using nuclear DNA, the construction of a phylogeny for North American wapiti to identify lineages and confirm their taxonomic classification, the establishment of a d-loop data-bank to produce more robust estimates of divergence; and to confirm the genetic diversity of populations presently believed to be homogenous. From a more global standpoint, the relationship between Old World and New World mammals, could also be concluded. These inquiries, however, can be extended to any species, subspecies or population, where the relationship between groups is questionable, and the genetic diversity appears to be minimal or non-existant.

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Appendix A

Additional populations and herds that were important to the conservation of bison in North America.

Banff National Park, Alberta Banff received two bison cows and one bull from the Texas Goodnight herd as a gift of T.G. Blackstock, a Toronto lawyer, in 1897 and 13 bison from Lord Strathcona of Manitoba in 1898 (Novakowski 1989). By 1899, the bison herd in the first Canadian park numbered 31 animals (Ogilvie 1979). In 1904, Banff traded two of their bison for two Corbin bulls. The population prospered and in 1909, 77 animals were moved to Buffalo National Park at Wainwright. Another 14 were moved in 1914 (Novakowski 1989). This left only 20 bison in the park in 1929. Therefore, Banff was sent 15 bison of Pablo-Allard origin from Elk Island National Park in 1931, followed by four more bulls, which arrived in 1946, 1951, 1955 and 1960. The plains bison in Banff were moved to a nearby Blackfoot reservation in 1980 and replaced with wood bison from Elk Island National Park.

Buffalo National Park, Alberta From 1909-1912, 316 bison from Pablo, 30 bison from Conrad Ranch, 77 bison from Banff, and 325 bison from Elk Island National Park were sent to Buffalo National Park at Wainwright (Federal Review 1990). Mosson Boyd of Bobcaygeon, Ontario, who had secured a few bison in 1896, bred hybrids, then quit and shipped all his stock to the Buffalo National Park in 1905 (Haines 1970). In 1914 ten bison were shipped from BNP to Buffalo National Park (Novakowski 1989). Between 1925-1928, 6,673 bison were shipped from Buffalo National Park to Wood Buffalo National Park (Federal Review, 1990). Buffalo National Park was shut down in the 1940's.

New York Zoological Gardens, New York The New York Zoological Gardens opened in November 8, 1899, and had seven bison: three bulls and one cow from the Goodnight herd plus one calf, one bull, and one cow from Oklahoma. These animals arrived at the park on October 1, 1899. The following year, the park also recieved two bulls from Whitney and three cows on loan from David Gardiner. In 1903, another 13 animals from Wyoming and three from the Whitney herd of October Mountain, Massachusetts were placed in the NYZG (Bridges 1974). The NYZG purchased three cows and one bull from the Page Wire Fence Company in 1904.

Appendix B

Wood Buffalo National Park, as illustrated in the Federal Environment Assessment Review of the Northern Diseased Bison (1990).